# Towards Increased Understanding of Stress-related Depression and its Treatment: Neurobehavioural Studies in Mice

---

**Dissertation**

**zur**

**Erlangung der naturwissenschaftlichen Doktorwürde (Dr. sc. nat.)**

**vorgelegt der**

**Mathematisch-naturwissenschaftlichen Fakultät**

**der**

**Universität Zürich**

**von**

**Damiano Azzinnari**

**von**

**Niederlenz AG**

**Promotionskomitee**

PD Dr. Christopher R. Pryce
(Vorsitz und Leitung der Dissertation)

Prof. Dr. med. Erich Seifritz

Prof. Dr. Hanns Möhler

Prof. Dr. Markus Rudin

**Zürich, 11.2.2014**

# Summary

Depression is one of the most prevalent human diseases, and causes high disability and mortality, both alone and in association with several other diseases with which it is highly co-morbid. Depression is a heterogeneous neuropsychiatric disorder with core symptoms of depressed mood, loss of interest/pleasure and fatigue, and a broad range of additional symptoms.

Human clinical and experimental studies demonstrate associations between environmental stress, the inflammatory system and depression. This evidence has led to the inflammation hypothesis of depression, which postulates that peripheral and central immuno-inflammation is increased by stress and induces neurobiological changes leading to depression. To test this hypothesis, animal models would be essential, but only if these are valid, meaning that they must comprise human-relevant components in terms of: (1) an environmental manipulation that causes psychosocial stress and induces (2) behavioural effects that are relevant to depression psychopathologies and are underlain by (3) inflammatory-neurobiological effects that are relevant to depression aetio-pathophysiology. This PhD thesis presents such a valid mouse model, constituting psychosocial stress in the form of chronic social defeat (CSD) – and behavioural changes in the form of increased fear of aversive events, generalised helplessness and fatigue. Subsequent identification of physiological and cortico-limbic transcriptome-expression characteristics of the model put the focus on immuno-inflammation-dopamine (DA) dysfunction as a major depression aetio-pathophysiology and treatment target.

Depression aetiology is proposed to include an important genetic component in the form of polymorphisms that predispose to depression aetio-pathophysiology in response to environmental stressors. The best-studied gene-environment interaction is that of individuals with genotype-dependent low serotonin (5-HT) transporter (5-HTT) function exhibiting increased depression prevalence if they experienced psychosocial stress during development (but not adulthood). In adulthood, low 5-HTT function is associated with increased immune resilience. Using a 5-HTT knockout mouse strain, interaction between 5-HTT genotype (heterozygous knockout vs. wildtype) and adulthood CSD was studied in terms of fear learning, peripheral immune response and brain 5-HT and DA activity. Relative to wildtype mice, heterozygous 5-HTT-control mice exhibited increased 5-HT and DA activity in the nucleus accumbens, with no effect of CSD. Heterozygous 5-HTT knockout inferred resilience against CSD in terms of attenuated fear and immuno-inflammation responses, providing evidence that the increased immune resilience associated with low 5-HTT function could extend to depression resilience in adulthood.

In terms of antidepressant treatment, recent evidence indicates that a single, low-dose infusion of racemic (RS-)ketamine exerts a rapid, sustained anti-depressant effect in otherwise treatment-resistant depressed patients. Stimulated by this, several mouse studies have reported that acute low-dose RS-ketamine exerts antidepressant effects in screening tests such as the forced swim test (FST). A study was conducted comparing effects of RS- and the more potent S-ketamine at low- and high-dose in the FST and on prefrontal cortex (PFC) glutamate release. Only low-dose RS-ketamine was effective in the FST and this effect, in contrast to a common assumption, was not

underlain by increased PFC glutamate release, which occurred with high-dose ketamine only. This evidence has important implications for the future development of antidepressant treatments based on ketamine's mechanism of action.

By combining environmental, genetic and pharmacological approaches to the translational study of depression psychopathology, aetio-pathophysiology and psychopharmacology, this PhD thesis presents several significant advances in (1) understanding of depression aetio-pathophysiology and (2) identification of novel antidepressant mechanisms-of-action.

# Zusammenfassung

Die Depression ist eine der häufigsten Krankheiten und führt oft zu Arbeitsunfähigkeit, Komorbidität und Tod. Neben den Hauptsymptomen depressive Stimmung, Interessensverlust und gesteigerter Ermüdbarkeit treten verschiedene zusätzliche Symptome auf, die Depression zu einer heterogenen neuropsychiatrischen Erkrankung machen.

Klinische und experimentelle Studien haben Zusammenhänge zwischen Umweltbelastung, Entzündungsreaktion und Depression aufgezeigt. Diesen Befunden liegt die Entzündungshypothese der Depression zu Grunde: Stress erhöht entzündungsstimulierende Reaktionen im Blut und Gehirn und dies verursacht wiederum neurobiologische Veränderungen, die die Erkrankung auslösen. Um solche Hypothesen zu testen, sind aussagekräftige Tiermodelle essentiell, welche aus drei der Depression analogen Komponenten bestehen müssen. (1) Ursache: Umweltbelastungen, die psychosozialen Stress herbeiführen; (2) Auswirkung: Verhaltensänderungen, die relevant zu den Psychopathologien der Depression sind; und (3) Mechanismus: neurobiologische Entzündungsreaktionen ähnlich zur Ätio-Pathophysiologie von depressiven Patienten. Diese Dissertation präsentiert ein valides Tiermodell bestehend aus einer Manipulation mit psychosozialem Stress, genannt „Chronic Social Defeat“ (CSD), und den daraus resultierenden Verhaltensänderungen in Form von erhöhter Furcht gegenüber aversiven Ereignissen, generalisierter Hilflosigkeit und gesteigerter Ermüdbarkeit. Zusätzlich wurden physiologische und cortico-limbische Veränderungen identifiziert, die den Fokus für die Ätio-Pathophysiologie und für mögliche Behandlungsmethoden auf eine Funktionsstörung im Immun-Entzündung-Dopamin(DA)-System richten.

Die Krankheitsursache von Depression beinhaltet eine wichtige genetische Komponente: Polymorphismen, die in der Anwesenheit von Umweltbelastungen eine Veranlagung zu Depression begünstigen. Eine viel zitierte Gen-Umwelt-Interaktion basiert auf einer genotyp-abghängigen, reduzierten Serotonin(5-HT)-Transporter(5-HTT)-Funktion. Individuen eines solchen Genotyps weisen eine erhöhte Prävalenz von Depression auf, falls sie psychosozialem Stress während ihrer Entwicklung, nicht aber im Erwachsensein ausgesetzt waren. Eine reduzierte 5-HTT Funktion ist im Erwachsenenalter mit einer erhöhten Immun-Resilienz assoziert. Basierend auf diesen Befunden wurde mit Hilfe einer 5-HTT-Knockout-Mauslinie die Interaktion zwischen dem 5-HTT-Genotyp und CSD im Erwachsenenalter auf Furchtverhalten, peripherer Immunantwort und zentraler 5-HT- und DA-Aktivität geprüft. Im Verhältnis zum Wildtyp haben die 5-HTT heterozygoten Mäuse eine erhöhte Aktivität von 5-HT und DA im Nucleus Accumbens. Des Weiteren zeigen die Heterozygoten nach der CSD Manipulation eine höhere Belastbarkeit in Form von abgeschwächter Furcht- und Immunentzündungsreaktion. Die erhöhte Immunresilienz, die mit reduzierter 5-HTT-Funktion assoziert ist, könnte zu einer Depressionsresilienz führen.

Neueste Studien haben gezeigt, dass eine akute, niedrig dosierte Infusion von racemischem (RS-) Ketamin zu einem schnellen, anhaltenden antidepressiven Effekt in der Behandlung von resistenten, depressiven Patienten führen kann. Inspieriet durch diese Befunde haben verschiedene Forschungsgruppen berichtet, dass akute, niedrig dosierte RS-Ketamin-Administrationen einen antidepressiven Effekt in Screeningmassnahmen wie dem „Forced Swim Test“ (FST) in der Maus

aufzeigen. Die Untersuchungen in dieser Dissertation konzentrieren sich auf den Vergleich der Auswirkungen auf das Verhalten im FST und auf die Abgabe von Glutamat im präfrontalen Cortex (PFC) bei akuter, niedrig und hoch dosierten RS- und S-Ketamin-Administrationen. Nur die niedrig dosierte RS-Ketamin-Dosis zeigte einen Effekt im FST, dies war jedoch unabhängig von Glutamat, da dessen Konzentration im PFC nur durch die beiden Hochdosierungen anstiegen. Diese Ergebnisse bringen wichtige Aspekte für die Entwicklung von neuen Antidepressiva mit ähnlichem Ketamin-Wirkungspotential zu Tage.

Diese Dissertation bringt mit Hilfe von umweltbedingten, genetischen und pharmakologischen Ansätzen zwei bedeutende Fortschritte für die translationale Forschung in der Psychopatholgie, Ätio-Pathopyhsiolgie und Psychopharmakologie von Depression: zum einen bewirkt sie ein besseres Verständnis für die Ätio-Pathophysiologie von Depression und zum anderen hilft sie bei der Ermittlung von Wirkungsmechanismen für neue Antidepressiva.

# Table of contents

# General introduction

## 1.DEPRESSION

### 1.1 Prevalence, symptoms and burden

Major depressive disorder (hereafter referred to as depression) is one of the most prevalent mental diseases, and is associated with disability, comorbidity to several other diseases, and mortality (Wittchen et al., 2011). It is a high risk factor for suicide and is a major reason of death worldwide (Holma et al., 2010). With its high lifetime prevalence (8 – 15 %), co-morbidity, treatment resistance and relapse, depression burdens the health care, mortality and workplace costs of Europe and the United States immensely (Richards, 2011). Depression is a heterogeneous psychiatric disorder with a broad range of symptoms which are described in two international medical classification systems: The Diagnostic and Statistical Manual of Mental Disorders, 5$^{th}$ Edition (DSM-5, 2013) and The International Classification for Diseases and Related Disorders, 10$^{th}$ Edition (ICD-10, 1994). In ICD-10, the symptoms are classified into core symptoms of depressed mood, loss of interest and fatigue, and common symptoms of disturbed sleep, poor concentration, low self-confidence, altered appetite, slowing of movements, self-blame and thoughts of suicide. To diagnose depression, at least two core and three common symptoms have to persist for at least two weeks.

To understand the psychopathology of depression, the symptoms such as depressed mood, loss of interest and fatigue must be first well defined and understood. Relevant here is the concept of emotion, describing the psychological states that arise in response to neutral, aversive or pleasant environmental stimuli, and are mediated by the punishment and reward systems of the brain. By determining the punishment and reward systems as the bases of mood, depressed mood can be seen as hyper-sensitivity of the punishment system, and loss of interest, and to some extent fatigue, as hypo-sensitivity of the reward system (Pryce and Seifritz, 2011). Clinical studies have shown that depressive patients exhibit increased emotional reactivity to aversive stimuli (Disner et al., 2011), reduced reactivity to pleasant stimuli (Russo and Nestler, 2013), and reduced pleasure after physical activity (Craig et al., 2006).

### 1.2 Neuropathology

Using *in vivo* brain imaging procedures including magnetic resonance imaging (MRI) and positron emission tomography (PET) to compare depressed patients with matched healthy controls, specific regions of the central nervous system (CNS) have been identified to exhibit altered functional and resting-state activity, altered volume and modified resting-state connectivity between regions (Price and Drevets, 2010). These studies indicate an increased processing of negative stimuli and a decreased processing of positive stimuli in depressive patients compared to healthy controls (Disner et al., 2011). For example, the functional activity of the rostral anterior cingulate cortex (ACC), a brain region central to attention, and of the amygdala (AMYG), a brain region central to emotional process, are relatively elevated in individuals with depression when aversive stimuli are presented (Drevets,

2001; Elliott et al., 2002; Mitterschiffthaler et al., 2008). Both results show that depressive patients are more likely to attend to and process aversive stimuli leading to a stronger experience of the negative event (Disner et al., 2011). This hypothesis is also supported by the fact that decreased reactivity to negative stimuli and decreased resting-state activity have been observed in the left dorsolateral prefrontal cortex (PFC) of depressive patients, a region associated with cognitive control and with modulation of the emotional processing in the AMYG (Disner et al., 2011; Gotlib and Joormann, 2010; Siegle et al., 2002). Furthermore, reduced functional connectivity between the thalamus and the dorsal ACC was associated with depression, which indicates a reduced inhibitory modulation of the limbic system processing negatively valenced stimuli (Anand et al., 2005). In the case where rewards/positive stimuli are presented, depressive patients show a decreased activation in the nucleus accumbens (NAcc) and PFC (Pizzagalli et al., 2009). The reduced response of the NAcc is associated with decrease positive-valence labeling by the reward circuitry, and that of the PFC with decreased reward sensitivity (Disner et al., 2011).

## 1.3 Aetiology: genetic and environmental factors

Depression is likely due to genetic (G) and environmental (E) factors (Sullivan et al., 2000). A major aspect of the environmental factors that increase the risk of depression is psychosocial stress as associated with adverse life events, including loss of a social partner, chronic stress due to work and financial problems, and interpersonal conflict (Kendler et al., 2003). Early life stress such as child abuse or neglect, also induces a number of physiological and anatomical abnormalities associated with depression (Heim et al., 2010). Whether or not individuals that experienced psychosocial stress (adverse life events in early life or adulthood) develop depression could be attributed to interaction of the environment with genetically-determined traits (Caspi and Moffitt, 2006). Studies examining the genetic factors of depression have identified only a small number of genes (Pryce and Klaus, 2013). In a meta-analysis of genome-wide association studies, no single locus was found to be associated with depression (Sullivan and Consortium, 2013). Due to the very small contribution of polymorphisms in single genes to depression risk and also to the heterogeneity of the disease, the likelihood of identifying risk genes by genome-wide association study is extremely low even with large sample sizes. (Pryce and Klaus, 2013). A meta-analysis that included case-control studies for candidate genes identified six genes in which a polymorphism is associated with depression risk: dopamine (DA) active transporter (*DAT*), DA receptor D4 (*D4*), methylenetetrahydrofolate reductase (*MTHFR*), guanine nucleotide binding protein beta polypeptide 3 (*GNB3*), apolipoprotein (*APOE*) and serotonin (5-HT) transporter (*5-HTT*) (Lopez-Leon et al., 2008) (for more information on 5-HTT see section 3.2). There is increasing evidence that the interaction between genes and environment (GxE), including resultant epigenetic modifications, plays an important role in the aetio-pathophysiology of depression (Caspi and Moffitt, 2006). To-date, the most studied GxE interaction involves a polymorphism also identified in the case-control studies mentioned above; namely the *5-HTT* polymorphism and its interaction with aversive life events (see section 3.2). Other studies have demonstrated association with depression risk of early aversive life events in interaction with single-nucleotide polymorphisms in genes modulating the hypothalamic-pituitary-adrenal axis, such as corticotropin-releasing hormone

(CRH) receptor 1 (*CRHR1*) and FK506 binding protein 5 (*FKBP5*) (Bradley et al., 2008; Zimmermann et al., 2011).

## 1.4 Pathophysiology

**The monoamine hypothesis**

The monoamine hypothesis of depression, including the indoleamine 5-HT and the catecholamines norepinephrine (NE) and DA, states that depression is associated with a reduced availability of either one specific or a combination from the three monoamines (Richelson, 1996). Monoamines have a particular role in modulating depression-relevant forebrain regions with afferent input from the midbrain and brainstem: 5-HT from the dorsal raphe nucleus (DRN), NE from the locus coeruleus (LC) and DA from the ventral tegmental area (VTA). Their networks regulate negative- and positive-valence emotionality, awareness and executive function (Krishnan and Nestler, 2008). Since the serendipitous discovery of different antidepressants that act by increasing the availability of monoamines, in particular the selective 5-HT reuptake inhibitors (SSRIs) in the mid-1980s, 5-HT and its metabolic and signalling pathways have been in the focus of depression research (Mulinari, 2012). In addition to anti-depressant effects of SSRIs, further clinical evidence in support of the 5-HT hypothesis has been reported. Thus, *post mortem* studies in depressive patients identified decreased 5-HT metabolites in cerebrospinal fluid (CSF) and increased $5\text{-HT}_2$ receptor levels in blood cells. In *in vivo* studies, depression was associated with (a) decreased physiological blood levels of 5-HTT and the 5-HT precursor L-tryptophan; (b) a polymorphism of the 5-HTT gene; and (c) decreased brain-imaging activity of 5-HTT in mesolimbic regions (Saveanu and Nemeroff, 2012). In genetic mouse models in which receptors, transporters or enzymes belonging to the 5-HT pathway were deleted, depressive-like or depression-resilient behaviours have been demonstrated depending on the gene (Dominguez-Lopez et al., 2012). The action of SSRIs is to increase 5-HT transmission immediately, by inhibiting its degradation or reuptake; therefore, it is not understood why the treatment requires weeks for the improvement of mood (Szanto et al., 2003). Similar findings were also observed for the NE-ergic system; NE reuptake inhibitors increase NE availability and show anti-depressant effects (Saveanu and Nemeroff, 2012). Additionally, decreased levels of NE metabolites in CSF and increased density of β-adrenergic receptors in post-mortem brain tissue were found in depressed patients, suggesting a receptor up-regulation due to lower ligand concentration (Saveanu and Nemeroff, 2012). Due to the low remission rates of the NE and 5-HT reuptake inhibitors (NSRIs), a class of antidepressant acting on both monoamines, the third monoamine, DA, was also proposed to be important in the pathophysiology of depression. Reward anticipation and sensitivity are reduced in depression, and the mesolimbic DA-ergic pathway is a major neurotransmitter system in the reward circuit (Salamone and Correa, 2012). In animal studies, depletion, pharmacological modulation and electrical and optogenetic stimulation of the VTA impact on the responsiveness to reward stimuli (Russo and Nestler, 2013). In depressive patients, decreased CSF concentration of DA metabolites has been reported, as have reduced DA transporter binding sites and increased density of the D2/D3 receptors. Drugs increasing the availability of DA have also been identified to induce anti-depressant effects (Saveanu and Nemeroff, 2012).

**The glutamate hypothesis**

Glutamate is the major excitatory neurotransmitter of the brain. It has been associated with depression since the discovery of the antidepressant class of N-methyl-D-aspartate (NMDA) receptor antagonists in the early 1990's (Trullas and Skolnick, 1990). Elevated glutamate levels in blood, CSF and brain tissue of frontal cortex were found in depressive patients (Sanacora et al., 2008) and antidepressants such as fluoxetine and s-citalopram have been demonstrated to reduce the glutamate level in plasma of depressive patients (Kucukibrahimoglu et al., 2009). Brain imaging findings diverge depending on the brain region. Decreased glutamate binding was observed in ACC, whereas glutamate binding was increased in occipital and parietal cortices of depressive patients. These abnormal glutamate levels have been related to changes in glial cell function (Sanacora et al., 2008; Walter et al., 2009). In ACC, and also AMYG and hippocampus (HIPP), there is immunohistochemical evidence for reduced glial cell counts and density, and reduced glia-to-neuron ratios (Harrison, 2002). Glial cells are responsible for glutamate reuptake and conversion to glutamine that is transported back to the neuron and reconverted to either glutamate or GABA (Rajkowska and Miguel-Hidalgo, 2007). Expression of the glial glutamate transporter and the glutamate degradation enzyme glutamine synthetase is reduced in depression (Choudary et al., 2005). Supporting this hypothesis, animal studies have shown that early life stress or chronic adult life stress induce a reduction in astrocyte density in prefrontal cortex (PFC), HIPP and AMYG and a decrease in escape behaviour of controllable aversive stimuli and in reward sensitivity, whereas the glutamate-modulating drug riluzole reversed theses effects (Banasr and Duman, 2008; Leventopoulos et al., 2007). Acute and chronic stress also increased the extracellular glutamate level and reduced glia mediated glutamate cycling in limbic and cortical areas (Sanacora et al., 2008). Recent pharmacological findings in human studies support the glutamate hypothesis of depression: ketamine, an NMDA glutamate receptor antagonist exhibits rapid and prolonged therapeutic efficacy in treatment-resistant depressed patients (see section 4.3 to 4.6).

**The HPA axis hypothesis**

For many decades, the hypothalamic-pituitary-adrenal (HPA) axis was regarded as *the* physiological signaling pathway responsible for bodily responses to environmental stress. Stress perceived and processed by the brain induces an increase in intermediary neuropeptides (CRH, arginine vasopressin (AVP)) and hormones (adrenocorticotropic hormone (ACTH)) of the HPA axis resulting in high levels of cortisol in blood plasma (corticosterone in rodents). Together with findings in clinical studies, including that a majority of depressive patients exhibit higher levels of basal cortisol than those of healthy probands, and that Cushing's syndrome patients (a disease induced by prolonged exposure to hypercortisoleamia) exhibit high co-morbidity with depression, the hyperactivity of the HPA axis was hypothesized to induce and maintain depression (Saveanu and Nemeroff, 2012). The first intermediate neuropeptide, CRH is secreted by the hypothalamus into the hypothalamo-hypophyseal portal system, and is also a neurotransmitter. Levels of CRH are increased in the CSF and in cerebrocortical areas of depressive patients (Merali et al., 2004). The observed decreased density and decreased mRNA expression of the CRHR in the frontal cortex of depressive patients suggest down-regulation due to hyper-secretion of CRH in depression (Merali et al., 2004). In animal studies, administration of CRH directly into the brain induces depressive-like behaviors (Heinrichs et al., 1995). Two neuroendocrine

challenge tests of the reactivity of the HPA axis for clinical and animal research purposes have been developed: (a) The dexamethasone (DEX) suppression test (DST), in which DEX administration inhibits the secretion of the second mediating hormone ACTH, thereby suppressing the release of cortisol or corticosterone. (b) The CRH test in which administration of synthetic CRH increases levels of ACTH and cortisol/corticosterone. In the majority of depressive patients, both reactions have been shown to be disrupted: DEX suppression of cortisol is reduced, as is synthetic CRH stimulation of ACTH. Reduced DEX suppression is consistent with an impaired corticosteroid receptor negative feedback which is also consistent with the increased levels of basal cortisol in some depressive patients (Saveanu and Nemeroff, 2012). The challenge test considered to be the most effective as a biomarker of depression is the combined DEX/CRH test (Ising et al., 2005). Drugs blocking receptors of the HPA axis such as the CRHR1 and glucocorticoid receptor have been tested (see section 4.2).

**The cytokine hypothesis**

Recently, evidence has been obtained that associates depression with activation of the immune system (Dantzer et al., 2011; Maes et al., 2009; Miller et al., 2009). These data include various clinical studies demonstrating: (a) the increase of pro-inflammatory cytokines e.g. tumor necrosis factor (TNF) and interleukin(IL-)6 in blood plasma of depressed patients (Dowlati et al., 2010), (b) the association of the severity of depressive symptoms with the increased level of cytokines (Raison et al., 2010), (c) the anti-inflammatory effect of antidepressants (De Berardis et al., 2010), (d) the reduction of depressive symptoms by the TNF antagonist etanercept (Tyring et al., 2006), (e) the induction of depression-relevant psychological states by immune-stimulation with endotoxin, typhoid vaccine or interferon alpha (INF-α (Capuron and Miller, 2004; Eisenberger et al., 2009; Harrison et al., 2009), (f) the co-morbidity of depression with autoimmune diseases such as multiple sclerosis and diabetes mellitus (Dalton and Heinrichs, 2005; Nouwen et al., 2010), and (g) the association of polymorphism in the TNF gene with depression (Bosker et al., 2011). Stress such as early life stress or chronic aversive life events lead to chronic activation of the immune system. Diverse pro-inflammatory cytokines, e.g. IL-6 or C-reactive protein (CRP), have been reported to be increased in couples after divorce and in adults after childhood maltreatment (Saveanu and Nemeroff, 2012).

In mice, repeated social stress leads to increased cytokines level in blood (Savignac et al., 2011) and administration of pro-inflammatory cytokines (e.g. IL-1β and TNF) induces depressive-relevant behaviors such as social avoidance and decreased motor activity (Dantzer, 2001). Peripheral cytokines have been demonstrated to enter the CNS via transporters in the blood brain barrier, and to act peripherally on cytokine-producing monocytes which themselves traverse the brain parenchyma (Banks, 2006; D'Mello et al., 2009). Pro-inflammatory cytokines modulate other neuronal functions implicated in the pathophysiology of depression (see sections above): (a) activation of 5-HTT leading to decreased synaptic availability of 5-HT (Zhu et al., 2006), (b) increased expression of DA transporter and decreased production of DA leading to decreased synaptic availability of DA, (c) activation of glutamatergic signaling, (d) increase in CRH production leading to a hyperactivity of the HPA axis, and (e) reduction of hippocampal neurogenesis (Felger and Miller, 2012; Saveanu and Nemeroff, 2012).

Current and promising aspects of research in depression link the aetiological aspect of cytokine hypothesis with pathophysiological states of depression, such as the 5-HT and DA deficiency (Felger and Miller, 2012; Maes, 1995). The essential amino acid tryptophan is the precursor of 5-HT and it determines the rate of biosynthesis of 5-HT. Tryptophan is either processed to 5-hydroxytryptophan which is then converted to 5-HT, or to kynurenine (KYN) which is then metabolized to kynurenic acid (KA) or quinolinic acid (QA). For the metabolism of KYN two enzymes have been identified, tryptophan-2,3-dioxygenase (TDO) and indoleamine-2,3-dioxygenase (IDO). IDO is activated by cytokines such as TNF and INF-α (Dantzer, 2009). This activation may be responsible for a cytokine-induced reduction of 5-HT production, shifting the tryptophan metabolism to KYN and then KA and QA. This hypothesis is supported by many studies: IDO is present in macrophages and dendritic cells of the brain, and activity of IDO is increased during acute and chronic immune system activation (Wirleitner et al., 2003). In mice, systemic administration of the immune system-stimulating lipopolysaccharide (LPS) increases the level of IFNɣ in the periphery and activates IDO in the periphery and in brain (Lestage et al., 2002). However, there is no consistent evidence for a change in 5-HT turnover by IDO. There are several reported effects of this kynurenine pathway:(a) the antagonistic or agonistic effects on the NMDA receptor of KA and QA, respectively, altering glutamate homeostasis and (b) the neurotoxic effect of QA activating nitric oxide agents (Felger and Miller, 2012). The latter induces oxidative stress in neurons, reducing the activity of tetrahydrobiopterin (BH4), an essential co-factor for tyrosine hydroxylase, the rate-limiting enzyme for the conversion of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) which, in a further enzymatic step, is metabolized to DA. Two other pathways in which cytokines reduce the DA availability in synaptic clefts are: (a) reduction of the expression of vesicular monoamine transporter 2 (VMAT2) which is responsible for the cytosolic package of DA in vesicles ready for release, and (b) increase in the expression of DAT (Felger and Miller, 2012). The strength of the cytokine hypothesis is its interface capacity between various pathophysiological states of depression.

# 2. ANIMAL MODELS

## 2.1 Importance of animal models

A major reason that depression research has, in the last decades, failed to provide evidence for novel pathophysiological mechanisms and antidepressant targets is the lack of valid animal models for depression. Animal models with aetiological, face and construct validity would allow for translational research in the form of bi-directional transfer of clinical aetio-pathophysiological knowledge of depression to animal models and the transfer of structural, neurochemical, molecular and neuropharmacological information in animal models back to depressed patients (Pryce and Seifritz, 2011). Animal studies are essential to test cause-effect hypotheses for the aetio-pathophysiology of stress-related depression and for identification of molecular targets for development of novel effective treatments. Establishing such an animal model requires three basic components: (1) genetic and/or environmental manipulation(s) of aetiological relevance to depression, (2) behavioural assays of relevance to depression neuropsychopathology, and (3) physiological effects analogous to those in depression. If well-validated, animal models, especially rodent models, can be widely used to question the neurobiological and genetic basis of depression.

## 2.2 Validity of animal models

To define the quality of an animal model in neuropsychiatric disorders, four validity criteria have been identified: aetiological, face, construct and predictive validities. Aetiological validity refers to the manipulation of the animal, that is, the factors that predispose to and trigger the disease-relevant changes. The aetiological process of the disease in human should be mimicked as accurately as possible in the animal. Chronic psychosocial stress is a major aetiological factor for depression (Kendler et al., 1999) and therefore chronic social defeat (CSD), the mouse model for depression presented in section 2.3 and in Project A and B, has potential for high aetiological validity. Face and construct validities refer to the similarities of the manipulation-induced changes to the human symptoms and pathophysiological states, respectively. Anatomical, biochemical and behavioural features in the animal must coincide with the impairment of neuropsychiatric patients. The last validation type, predictive or pharmacological validity, demands that the face- and construct-valid effects observed in the model are reduced by treatments specific to the disorder i.e. antidepressants in the case of depression. For example, one of the few current rodent models with aetiological-face-predictive validity is the chronic mild stress (CMS) procedure (Willner, 2005). It comprises prolonged exposure to various physical and social stressors (aetiological validity). These factors induce a gustatory anhedonia (face validity) which can be reversed by imipramine (predictive validity) (Monleon et al., 1995). Recently, it has been demonstrated that CMS mice exhibit reduced bursting activity of DA neurons and that optogenetic stimulation of firing by DA neurons in the VTA reverses CMS-induced gustatory anhedonia (Tye et al., 2013). These last results imply also a high construct validity of this model.

## 2.3 Social stress as a manipulation with aetiological validity in animal depression models

Social stressors are aetiological factors in human depression (Kendler et al., 1999). In animal research, social stress has been used as a manipulation in depression models in various forms. These include social isolation (Bartolomucci et al., 2003), exposure to ex-resident odours (Rodgers and Cole, 1993) and experiencing repeated disruption of social networks (Schmidt et al., 2010). In the last 15 years, CSD, consisting of a direct attack session between a dominant and a submissive mouse, has gained interest as a manipulation with aetiologcal validity for a depression model. Koolhaas and colleagues demonstrated in mice that social defeat induces higher stress responses in terms of increased corticosterone level in plasma relative to other stressors including foot shock or forced swim sessions (Koolhaas et al., 1997). Even a single defeat was enough to sensitize the mouse to have a higher response to subsequent minor stressors. The first CSD procedure used BL/6 mice as aggressors and subjects (Kudryavtseva et al., 1991). Subsequent modifications were conducted to use ex-breeder CD-1 strain mice as aggressors (Golden et al., 2011). The readout effects were increased motor retardation measures such as decreased activity in an open field test and in a forced swim test (FST) (Kudryavtseva et al., 1991), decreased reward sensitivity in the sucrose preference test (Yu et al., 2011) and increased anxiety in the elevated plus maze test (Avgustinovich et al., 2005; Krishnan et al., 2007). A major focus of behavioural effects of CSD is the induced social avoidance. It is measured using the social approach-avoidance test, consisting of two sessions in an arena containing a small wire-mesh cage: in the first session, the subject mouse explores the arena with the cage empty and in the second session with the cage containing a novel CD-1 mouse; relative to controls, CSD mice exhibit decreased time in proximity of the cage in the second session, i.e. high social avoidance. It is noteworthy that such social avoidance tests use the same category of aversive stimulus as for CSD; accordingly, a stimulus-specific effect of CSD is being demonstrated and high avoidance of this specific stimulus can even be considered as adaptive behaviour.

## 2.4 Behavioral readouts with face validity in depression animal models

Each of the three tests described below provides an example of measuring objective, face-valid animal behavior with respect to symptoms of depression.

**Fear conditioned freezing**

Fear conditioning involves the organism acquiring an emotional response to a neutral stimulus (light, tone and context) that predicts an innate aversive stimulus (e.g. painful electroshocks). In rodents fear can be measured as freezing behaviour to inescapable aversion. This form of fear conditioning and responding is mediated primarily by the basolateral and central nuclei of the AMYG (LeDoux, 2000). Human studies of emotional reactivity are typically not based on the same behaviour such as freezing in the classical conditioning, rather psychopathological scales are used and functional MRI to measure regions-specific reactivity to images of happy, neutral or sad faces (Blanchard et al., 2001).

**Learned helplessness**

The concept of learned helplessness (LH) is a major theory for both onset and maintenance of depression (Abramson et al., 1978; Pryce et al., 2011). LH comprises the experiencing of an

uncontrollable stressor with emotional valence leading to a generalized perception of aversive events as uncontrollable, even if they are not (Pryce et al., 2011). In animal studies, the two-way shuttle arena is used for measuring the deficit in avoid or escape behaviour. Some of the first LH experiments were performed in rats: subjects received either uncontrollable or controllable tail electroshocks which could be terminated by turning a wheel and were tested with controllable, escapable foot electroshocks by another operant behaviour in another context on the following day (Weiss and et al., 1981). In mice, the pre-exposure day and the test have been performed in the same context and demanding the same operant behaviour to control the foot electroshock ((Anisman and Merali, 2001; Pryce et al., 2012) see Appendix). In both rodent tests, animals that have experienced uncontrollable electroshocks, compared to those that experienced controllable electroshocks, exhibit a deficit in escapes in the test phase. Pharmacological studies have demonstrated that by inactivating the PFC, rats that experienced controllable electroshocks exhibit a deficit in escape, and by activating the PFC, rats that experienced uncontrollable electroshocks exhibit normal escape behaviour (Amat et al., 2005). These results indicate an important role of the PFC in mediating controllability and helplessness. In human, the tests were adapted from the animal paradigms and typically also use inescapable versus escapable electroshocks.

**Fatigue during an effortful physical task**

Despite being a core symptom of depression, fatigue is poorly understood and has not been studied in animal models of depression. In psychopathological scales and tasks, human fatigue is commensurate with decreased motivation and perceived ability to maintain a level of physical or mental effort (Hossain et al., 2003; Keller et al., 2007; Weinstein et al., 2010). Self-reported fatigue is associated with increased glucose metabolic activity in NAcc and putamen (Capuron et al., 2007).

# 3.GENE- ENVIRONMENT INTERACTION

## 3.1 Genes

**The example of 5-HTT polymorphisms in human**

Within the promoter sequence of the 5-HTT gene, the 5-HTT gene-linked polymorphic region (5-HTTLPR), either a short (*s*) or a long (*l*) nucleotide sequence is present. The *s*-allele is associated with lower transcriptional efficacy leading to decreased 5-HTT expression and decreased 5-HT reuptake in the synaptic cleft and, paradoxically given that 5-HT reuptake constitutes current first-line antidepressant therapy, increased risk of depression (Murphy and Lesch, 2008). PET imaging studies using radiolabelled RTI-55, a DA- and 5-HT transporter ligand, demonstrate a functional correlate of the three genotypes: *s/s* and *s/l* individuals exhibit a decrease in 5-HTT binding sites compared to *l/l* individuals (Murphy and Lesch, 2008). MRI studies in healthy controls have shown that *s*-carriers exhibit increased activity in AMYG in response to fearful faces compared to *l*-carriers (Canli et al., 2006; Hariri et al., 2002). These findings are not in concordance with the 5-HT deficiency hypothesis of depression nor with the SSRI mechanism of action.

## 3.2 Environment

**Early Life Trauma**

Childhood maltreatment consisting of emotional and physical neglect or physical and sexual abuse are common in our society. Three million cases of child maltreatment are reported annually, and a considerable number of abuse and neglect cases are not registered (Saveanu and Nemeroff, 2012). Many studies have shown a marked association between aversive life events in children and the onset of depression in adulthood. For example, women that experienced sexual abuse as a child compared to women with no abuse have a four-fold increased risk of developing depression (Mullen et al., 1996). Early life stress also changes the course of depression: it reduces age at first episode onset, prolongs episodes, and decreases the rates of remission and recovery (Saveanu and Nemeroff, 2012). However, not all maltreated individuals develop depression, and three possible explanations for such resiliency are (a) perceived positive parental care and the quality of relationship to peer/partner i.e. social support, (b) timing of the aversive events, and (c) genetic predisposition.

**Adulthood aversive life events**

Stressful situations during adulthood i.e. aversive life events are associated with depression and are usually categorized as social loss (e.g. death of a loved one, separation from social partner), personal insecurity (e.g. personal failure, interpersonal conflicts and insecurity over future), chronic stress (e.g. work, financial or legal problems) and health problems (Keller et al., 2007). The different groups correlate differently with specific depressive symptoms. The social loss component is most associated with a high level of sadness, appetite loss and anhedonia, and the chronic stress component with fatigue and hypersomnia (Keller et al., 2007). As mentioned for early life stress, aversive life events

are also not sufficient to induce depression in all individuals and not all depressive patients show a history of aversive life events. Similar explanations may play a role as described above.

## 3.3 Gene x Environment interaction

The first (and most robustly demonstrated) GxE interaction was described by Caspi and collegues (Caspi et al., 2003). In this study an association between the *s*-allele of the 5-HTTLPR of the 5-HTT gene and stressful life events in childhood or young adulthood was demonstrated. The effects of adult stressful life events between the ages of 21 and 26 on depressive symptoms were significantly higher among *s*-allele than homozygous *l*-allele carriers. Aversive life events experienced in the same time period also increased depressive symptoms and predicted a diagnosis of depression in *s*-allele individuals compared to *l*/*l*-allele carriers (Caspi et al., 2003). Another study demonstrated that *s*/*s* individuals compared to *l*-allele carriers were more vulnerable to develop depression after aversive life events (Kendler et al., 2005). Taken together, these data suggest that the 5-HTT gene modulates the relationship between stressful events and depression aetio-pathophysiology.

## 3.4 Animal studies of GxE interaction

**The example of 5-HTT KO mice**

A genetic mouse model was created mimicking the effect of the 5-HTTLPR polymorphism in human: the 5-HTT partial knockout (5-HTT HET) mouse. By homologous recombination, a segment of the 5-HTT gene that contains the start codon and a sequence responsible for cellular transport was replaced causing an inactivation of gene transcription (Bengel et al., 1998). Compared to wild type (WT), HET mice show low 5-HTT levels and increased 5-HT levels in different brain regions (Bengel et al., 1998; Mathews et al., 2004). Behaviorally, a small number of studies have shown that HET compared to WT mice exhibit anxiogenic behaviors (in the dark-light box emergence test but not in the elevated plus maze, (Jansen et al., 2010), intermediate aggressive behavior (Holmes et al., 2003), increased acquisition of learned helplessness (Pryce et al., 2012), reduced sensitivity to non-reward in operant conditioning (Ineichen et al.,2012), and no altered response in fear conditioning acquisition and expression (Narayanan et al., 2011).

**Stress model in mice**

A number of stress mouse models have been developed for examining the effects of early and adult life stress (see section 2.3). The most common stress manipulation for early life events in rodents is maternal separation (Pryce and Feldon, 2003). In rats, the absence of warmth and food during maternal separation leads to behavioural and endocrine phenotypes in adulthood. For example, separating pups for 3 hours per day during the first two weeks after birth led to increased emotional behaviours and increased physiological response to stress in adulthood (Carlyle et al., 2012). For modeling adult life stress in mice, one of the most common procedures is the CSD paradigm (see section 2.3).

**GxE interaction in mice**

In a recent GxE interaction study, repeated stress exposure during adolescence (post-natal day 28-47) led to increased anxiety and decreased activity in WT and HET mice (Spinelli et al., 2013). In the

probabilistic reversal learning task, a test measuring feedback sensitivity, stressed HET relative to non-stressed HET and WT mice exhibited poorer performance. In depressed patients and adolescents homozygous for the s-allele and exposed to childhood adversities, poorer performance has been reported in a human version of this task (Murphy et al., 2003; Owens et al., 2012). When adult stress was applied using CSD, 5-HTT HET mice exhibited mildly increased freezing behavior during expression of conditioned fear and considerably impaired extinction of learned fear (Narayanan et al., 2011). Increased sensitivity to CSD in HET mice in terms of increased social avoidance has also been reported (Bartolomucci et al., 2010): in this model the same aversive stimulus is used for the manipulation and the readout test, the CD-1 mouse, thus the increased avoidance exhibited by HET can be considered as adaptive rather than depression-relevant. In terms of physiology, mouse studies of 5-HTT gene x CSD interaction effects have reported a stress-induced increase of corticosterone in plasma and corticosterone metabolites in faeces but no interaction with genotype (Bartolomucci et al., 2010; Jansen et al., 2010).

# 4. ANTIDEPRESSANTS

## 4.1 Different types of depression treatment

The efficacy of the current antidepressant drugs, mainly targeting the monoamine systems, is 50- 70% in terms of two drug trials of different mechanism-of-action and based on a chronic treatment regimen (Fava and Davidson, 1996; Little, 2009). Relapse and remission occur frequently. Patients resistant to pharmacotherapy are treated with psychotherapy and, if no improvement occurs, electroconvulsive therapy or deep brain electro-stimulation in the dysfunctional limbic-cortical circuit are options for such cases of treatment resistance. Different types of psychotherapy exist (e.g. cognitive behavioural therapy, interpersonal therapy, psychodynamic psychotherapy or supportive psychotherapy), but robust studies on their efficacy relative to placebo and differential efficacy are lacking (Saveanu and Nemeroff, 2012). . For deep brain stimulation, still in its infancy as a depression treatment, electrodes are placed surgically in the brain and provide electric impulses in depression-relevant brain areas. Stimulation of the NAcc has led to sustained remission of depressive symptoms (Schlaepfer et al., 2008). Because of the unknown action of deep brain stimulation and electroconvulsive therapy and their invasive methodology it is important to improve psychopharmacology and psychotherapy.

## 4.2 History of antidepressants and novel targets

In the 1950's, the serendipitous discovery of two classes of anti-depressant, monoamine oxidase (MAO) inhibitors and tricyclic antidepressants (TCA), both elevating the levels of monoamines in the brain, started the psychopharmacological era in psychiatry. In the 1940's, anti-histamines were studied and due to some anti-depressant side effects the first TCA, imipramine, was discovered. Similar findings were observed with the MAO inhibitors developed against tuberculosis. Iproniazid was approved in 1958, but was withdrawn due to its hepatotoxic effects. Since then, more specific MAO inhibitors have been developed. In the meantime, clinical studies assessed the importance of decreased 5-HT in depression and drugs designed to increase the 5-HT level were developed. In the 1970's, the first SSRI, fluoxetine (Prozac), was approved. More recently, drugs acting on NE and 5-HT transporters (e.g. SNRI) have been used to treat depression. The increasing use of buproprion, a NE and DA reuptake inhibitor, has turned the focus to the DA hypothesis.

At the beginning of the new millennium, the CRHR1 antagonist R121919 showed promising results but failed due to liver toxicity (Zobel et al., 2000). Due to the HPA hypothesis and supported by the high expression of the glucocorticoid and mineralocorticoid receptors (GR and MR) in depression-relevant brain regions such as AMYG and HIPP (Kennedy and Rizvi, 2009), modulators of these receptors have been developed and assessed for anti-depressant efficacy (Ising and Holsboer, 2007). Although phase 1 tests of the GR antagonist mifepristone yielded positive results (Kling et al., 2009), it failed in advanced phases.

There is a need for novel antidepressants with mechanisms-of-action based on goal-directed research. Based on evidence from different genetic, brain-imaging and neurochemical studies, antidepressants must be developed to act on the specific neural circuitry that is altered in depression, not disrupting healthy systems. The present therapeutic drugs in development can be broadly

categorized into five groups: (a) multiple monoamine agents such as triple reuptake inhibitors or melatonergic receptor agonists (e.g. agomelatine), (b) glutamate signalling modulators such as NMDA receptor antagonists (e.g. ketamine) and modulators of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and metabotropic glutamate receptors, (c) agents acting on neurotrophic factors such as brain derived neurotrophic factor (BDNF), (d) modulators of the immune system such as antagonists of pro-inflammatory cytokines pathways, and (e) agents acting on neuropeptides such as neurokinin (Kennedy and Rizvi, 2009). The clinical evidence for the most novel drugs is described in section 1.4.

## 4.3 Pharmacology of ketamine

The most common purpose of ketamine concerns its anaesthetic effect. Ketamine acts as a non-competitive antagonist on the ionotropic glutamate $Ca^{2+}$-channel of the NMDA receptor. It inhibits the receptor via the phencyclidine binding site (Hirota and Lambert, 1996). Ketamine modulates also other receptors such as opioid, muscarinic and adrenergic receptors (Quibell et al., 2011). The molecule possesses a chiral centre leading to the R- and S-enantiomers. However, the equal racemic (RS-) mixture of both enantiomers is the most used for clinical studies. S-ketamine has a five-fold higher affinity for the NMDA receptor, and it has twice the analgesic potency (Quibell et al., 2011). The liver metabolizes both chiral molecules rapidly (half life time of 3 hours, (Katalinic et al., 2013)).

## 4.4 Ketamine in humans

One of the most encouraging rapid antidepressant effects has been provided by ketamine in treatment-resistant patients. Since the first study in 2000 demonstrating the antidepressant effect (Berman et al., 2000), there have been many confirmatory studies. Intravenous subanaesthetic infusion of 0.5 mg/kg ketamine over 40 minutes in depressive patients led to significantly reduced scores on the Hamilton Depression Rating Scale (HDRS) within a few hours; this effect persisted up to day 3 (Berman et al., 2000) and day 7 (Zarate et al., 2006). A higher ketamine dose of 1.0 mg/kg was administered during anaesthesia in depressive patients undergoing surgery. Similar effects could be observed with the HDRS at 1 day post-surgery; the effects were no longer significant after 3 days (Kudoh et al., 2002). Ketamine also induces side effects, including positive, negative and dissociative symptoms of schizophrenia, and manic symptoms (Katalinic et al., 2013). There are few studies of the different effects of the two enantiomers. S-ketamine causes less psychotomimetic effects (Paul et al., 2009). Given the few studies to date, it is still unknown how application, dosage and the different enantiomers act on the brain and on depression. However, the results provide evidence that dysfunctional glutamate signalling and metabolism are important to the pathophysiology of depression.

## 4.5 Ketamine in mice

To understand the mechanism-of-action of ketamine in human, rodent studies provide translational support. High-dose administration of ketamine was related to increased glutamate release in medial PFC of rats (Moghaddam et al., 1997). No such study of neurotransmitter effects by low-dose ketamine has been reported so far. Studies analysing the quantification of cellular-pathway proteins indicate that ketamine effects may have a complex inverted U-shape dose-response relationship (Autry et al., 2011; Li et al., 2010). Several mouse studies report effects of acute low-dose RS-

ketamine (2.5-5.0 mg/kg ip) on behaviour in antidepressant screening tests e.g. the FST (Autry et al., 2011).

# 5. AIMS OF THE PHD THESIS

## 5.1 Aim of Project A (Chapter 2)

With respect to the aetiology of depression, the Project A of this PhD thesis examines the impact of stressful socio-environmental factors on physiology, behaviour and *ex vivo* brain transcriptome expression. The aim was to adapt the CSD procedure so that the physical wounding that occurs in the existing protocol was avoided, therefore: (1) the incisors of CD-1 mice were trimmed on a regular basis and (2) attack duration was timed to stop the session if the attacks exceeded the certain total time of attack. Under these refined conditions, CSD effects on physiological inflammatory markers were investigated. With respect to the psychopathology of depression, novel and face-valid behavioural readouts were developed and applied. These comprised measures of motoric, emotional, motivational and cognitive reactivity to uncontrollable or controllable aversive stimuli. Both short- and long-term effects of CSD were measured in these behavioural tests. Given that the innate aversive stimulus in each of these tests was painful electroshock, the extent to which experiencing chronic psychosocial stress led to generalized hyper-reactivity, including impaired control, to other stress stimuli was under study. Having confirmed that the psychosocial stressor of refined CSD induces depressant-relevant behavioural changes and induced depression-relevant peripheral inflammation, a hypothesis-free analysis of CSD-induced differential gene expression in cortico-limbic brain regions was conducted using next generation sequencing and identified de-regulation of immune-inflammation and DA genes.

## 5.2 Aim of Project B (Chapter 3)

In Project B, the mouse model validated in Project A was used to investigate the interaction of the same environmental stressor on a depressant-relevant genetic mutation. Based on the human evidences for the S allele of the 5-HTT promoter region possibly conferring adult stress resilience, the corresponding 5-HTT KO mouse line was under investigation. The aim was to find out whether and via which mechanisms 5-HTT genotype modulates the impact of adult stress on depression-relevant behaviour, immune function and DA-ergic signalling. The CSD x 5-HTT interaction effects on reactivity to aversive events were studied in terms of fear-induced freezing in a context conditioning test. That is, mouse model evidence was sought in support of both the 5-HT and cytokine hypotheses of depression based on their integrative regulation of brain and behaviour.

## 5.3 Aim of Project C (Chapter 4)

In this part of the PhD thesis, important issues raised by the translational evidence of acute low-dose ketamine as an antidepressant treatment were addressed. In non-manipulated mice, the following effects were investigated: (1) RS- or S-ketamine at equivalent low (3, 1.5 mg/kg) or high (50, 25 mg/kg) dose in the FST. (2) RS- or S-ketamine at equivalent low dose in the LH paradigm. (3) RS- or S-ketamine at equivalent low or high dose on glutamate release in mPFC. (4) RS- or S-ketamine at equivalent low or high dose on BDNF in HIPP. (5) RS- or S-ketamine at equivalent low or high dose on mTOR in HIPP and mPFC. Of particular interest was whether a low-dose of high-affinity S-ketamine

exerted the most robust effect in antidepressant screening tests and, if so, whether this was associated with increased mPFC glutamate release, increased BDNF levels and increased mTOR phosphorylation as recently hypothesized with the low-dose RS-ketamine administration.

# Chapter 2

**Mouse model for psychosocial stress-induced depressed mood and fatigue highlights inflammation-dopamine aetio-pathophysiology**

Damiano Azzinnari[1, 6], Hannes Sigrist[1], Simon Staehli[1], Rupert Palme[2], Tobias Hildebrandt[3], German Leparc[3], Bastian Hengerer[4], Erich Seifritz[5, 6], Christopher R. Pryce[1, 6]

1 Preclinical Laboratory for Translational Research into Affective Disorders, Department of Psychiatry, Psychotherapy and Psychosomatics, Psychiatric Hospital, University of Zurich, Switzerland

2 Institute of Medical Biochemistry, Department of Biomedical Sciences, University of Veterinary Medicine Vienna, Austria

3 Target Discovery, Boehringer Ingelheim Pharma GmbH & Co. KG., Biberach, Germany

4 CNS Diseases Research, Boehringer Ingelheim Pharma GmbH & Co. KG., Biberach, Germany

5 Department of Psychiatry, Psychotherapy and Psychosomatics, Psychiatric Hospital, University of Zurich, Switzerland

6 Neuroscience Center Zurich, University of Zurich and ETH Zurich, Switzerland

# 1. SUMMARY

Animal models of depression invariably lack adequate aetiological and face validity, and therefore relevance to much-needed identification of aetio-pathophysiological processes and molecular targets for novel antidepressant treatment. Overcoming these short comings requires environmental manipulations that confer chronic uncontrollable psychosocial stress without confounding physical injury, and objective behavioural readouts under emotional challenge focusing on core psychopathology domains such as generalised loss of control (helplessness) and fatigue. C57BL/6 mice were exposed to 15-day chronic social defeat with novel refinements that circumvented physical wounding. Effects were investigated in terms of: passive and active behavioural responses to controllable and uncontrollable electroshock; peripheral biomarkers of immuno-inflammatory and stress-hormone status; transcriptome-level gene expression status in hippocampus, amygdala and prefrontal cortex using next generation sequencing-canonical pathway analysis. Chronic psychosocial stress increased: fear of an uncontrollable negative event, as acquisition of freezing in the context of electroshocks; helplessness towards a controllable negative event, as reduced avoiding-escaping of electroshocks in 2-way active avoidance; psychomotor fatigue in the face of a controllable negative event, as reduced running on a moving treadmill to avoid-escape electroshock. Peripheral pro-inflammation was demonstrated by increased blood levels of tumor necrosis factor and interleukin-6, and splenomegaly. In a separate cohort, chronic psychosocial stress induced gene de-regulation in canonical pathways of immuno-inflammation and G-protein coupled receptors. In amygdala, gene de-regulation was consistent with compromised dopamine receptor 2 signalling. This novel mouse depression model with unusually high aetiological-face validity places the focus on stress-immuno-inflammation-dopamine dysfunction as a major depression aetio-pathophysiology and treatment target.

# 2. INTRODUCTION

Depression is a prevalent neuropsychiatric disorder with core symptoms of low mood, anhedonia and fatigue (DSM-5, 2013; ICD-10, 1994). It is generally accepted that the majority of current animal models for mental disorders, and certainly those for depression, are deficient in aetiological validity (manipulations that mimic human genetic and/or environmental depression risk factors and their interaction) and face validity (objective behavioural readouts of relevance to psychopathologies) (Hyman, 2012; Insel and Sahakian, 2012). Non-valid models and screening tests will not contribute to the much needed increased understanding of depression aetio-pathophysiology and identification of molecular targets for development of novel antidepressant treatments (Pryce and Seifritz, 2011). This paucity of valid models is particularly costly given the high resolution of the technologies available to study brain circuitry and cellular-molecular function (Hyman, 2012). For example, one of the few current rodent models with aetiological-face validity is gustatory anhedonia induced by chronic mild stress (CMS) comprising prolonged exposure to various physical and social stressors (Willner, 1997). Recently, it has been demonstrated that CMS mice exhibit reduced bursting activity of dopamine neurons and, using optogenetic and viral vector technologies, that optogenetic stimulation of firing by dopamine neurons in the ventral tegmental area reverses CMS-induced gustatory anhedonia (Tye et al., 2013). The important next step in such a model would be to identify the underlying aetio-pathophysiology and druggable molecular targets; precisely because of the aetiological-face validity there is a high probability that these would be clinically relevant.

Alongside the need for aetiological-face validity, when designing animal models of depression it is also essential to consider, *a priori*, hypotheses of depression aetio-pathophysiology. One hypothesis is that of immuno-inflammation, which proposes that psychosocial stress activates the inflammatory response pathway in the periphery, which in turn stimulates inflammation-related processes in the brain that substantially inhibit dopamine, serotonin and glutamate signalling (Haroon et al., 2012; Miller et al., 2009). Psychosocial stress is proposed as the major aetiological factor in the mouse environmental manipulation of chronic social defeat (CSD), which involves subject mice experiencing daily social subordinacy to aggressive dominant mice (Kudryavtseva et al., 1991). However, bite wounds are a common occurrence in the standard CSD protocol (Golden et al., 2011), and this of course confounds its aetiological validity as a mouse model for the psychosocial stress - immuno-inflammation hypothesis of depression. Therefore, in the present study, simple but effective refinements (also in the context of animal welfare 3Rs) of the CSD protocol were introduced such that psychosocial emotional stress was experienced in the absence of physical wounding.

With respect to behavioural readouts, like chronic mild stress, CSD has also been demonstrated to induce gustatory anhedonia (Krishnan et al., 2007). Another major focus has been that CSD mice exhibit passive avoidance of mice from the aggressor strain that induces defeat; whilst indicative of increased social fear, this is actually an adaptive fear to a specific punishing stimulus, rather than a pathological response (Krishnan et al., 2007). A major theory in depression aetiology and psychopathology is generalised helplessness: it proposes that a specific uncontrollable stressor of high aversive emotional valence (life event) is experienced (Kendler et al., 1999) and that this leads -

via neuropathological processes proposed to include deficient prefrontal cortical activity (Amat et al., 2005) - to a psychopathology of generalised environmental uncontrollability. Behaviourally, generalised helplessness is expressed as altered emotionality (aversive hyper-reactivity), motivation (deficient effort) and cognition (deficient action-goal expectancy) (Maier and Seligman, 1976; Pryce et al., 2011). We have recently established a face-valid paradigm of specific helplessness in which mice exposed to controllable electroshocks continue to escape, whereas mice exposed to the same electroshocks (duration, intensity) but now uncontrollable, develop deficient motivation to escape (Pryce et al., 2011). One major aim of the present study was to investigate whether CSD mice also develop an escape deficit to electroshock, which would constitute a novel mouse model of generalised uncontrollability/helplessness. Another major aim was to investigate the effect of CSD on psychomotor fatigue: although fatigue is a core symptom of depression (Demyttenaere et al., 2005), and although effortful-running fatigue has been studied in mice in the context of exercise physiology (Carmichael et al., 2006), to our knowledge this is the first study of an animal model of psychosocial stress-induced fatigue.

This novel mouse model - aetiologically and face valid for depression - was here applied to the study of aetio-pathophysiological processes: Firstly, translational physiological markers of immuno-inflammation (Dowlati et al., 2010; Maes, 2011) and glucocorticoid function (Rhen and Cidlowski, 2005) were obtained. Second, hypothesis-free transcriptome-level next generation sequencing and canonical pathway analysis were applied to investigate CSD de-regulation of gene expression in specific brain regions – namely, hippocampus, prefrontal cortex and amygdala – known to regulate the behavioural processes studied in the model (Amat et al., 2005; Maren et al., 2013) and to be functionally associated with depression psychopathology (Capuron et al., 2007; Price and Drevets, 2010; Strigo et al., 2008).

# 3.EXPERIMENTAL PROCEDURES

## 3.1 Experimental design

Three separate, inter-related experiments were conducted in C57BL/6 adult male mice to investigate the effects of chronic emotional psychosocial stress on behavioural reactivity to a different aversive stimulus, namely electroshock, presented in a comprehensive battery that allowed for the study of psychomotor activity, contextual fear conditioning, helplessness, pain sensitivity and fatigue. Physiological effects of CSD in these same mice were studied in terms of plasma tumor necrosis factor (TNF) and interleukin-6 (IL-6) levels and spleen and adrenal gland mass. In a separate cohort of CSD and control (CON) mice, transcriptome-level effects on gene expression in hippocampus (HIPP), prefrontal cortex (PFC) and amygdala (AMYG) were studied. Experimental designs are presented in Figure 1.

Experiment A (CON = 13, CSD = 14) investigated the short-term (days 15 to 17) effects of CSD on contextual fear conditioning, two-way active escape-avoidance and hot-plate pain sensitivity. At day 18, mice were killed and blood/tissues collected for study of effects of CSD on spleen and adrenal gland weight, plasma concentrations of pro-inflammatory cytokines and CORT.

Experiment B (CON = 10, CSD = 13) investigated the development (day -1 to 16) of effects of CSD on basal concentrations of faecal CORT metabolites, motor activity, water consumption and saccharin preference, the short- and long-term (days 17-18 and 29, respectively) effects of CSD on fatigue, and the long-term (days 30-32) effects of CSD on contextual fear conditioning and two-way active escape-avoidance.

Experiment C (CON = 12, CSD = 12) investigated the short-term (day 17) effects of CSD on region-specific transcriptome expression in fresh-fixed brain.

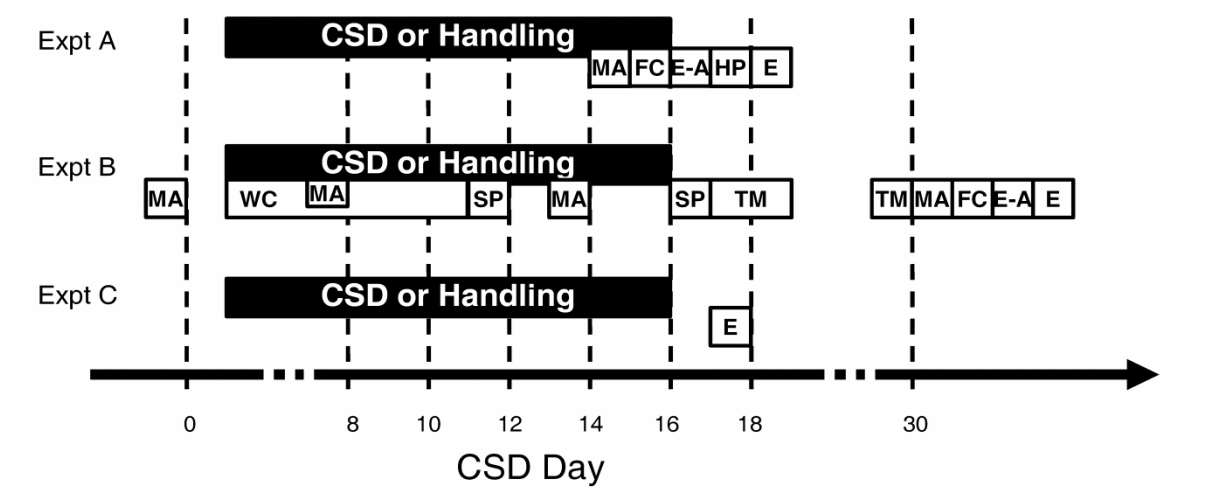



Figure 1: Experimental designs and aims of the three experiments. Experiment A investigated short-term effects of CSD (i.e. effects in the days immediately post-CSD) in the motor activity (MA) test, contextual fear conditioning (FC) test, two-way active escape-avoidance (E-A) test, and hot plate (HP) test. Experiment B investigated effects of CSD during the 15-day CSD in the water consumption (WC) test, the MA test and the saccharin preference (SP) test, the short-term effects of CSD in the SP test and the treadmill (TM) test, and the long-term effects of CSD (i.e. 2 weeks post final CSD day) in the TM, MA, FC and E-A tests. Experiment C investigated the short-term effects of CSD in otherwise non-manipulated mice on genome-wide gene expression in ventral hippocampus, amygdala, and medial prefrontal cortex. Euthanasia time point is indicated by E.

## 3.2 Animals and maintenance

Breeding of C57BL/6 mice was conducted in-house. Male offspring were weaned at age 3 weeks and caged in groups of 2-3 littermates. The study was conducted with a total of 76 mice born to 26 breeding pairs. Mice were aged 10-13 weeks and weighed 22.0-30.0 g at study onset. Male CD-1 mice (Janvier, France, www.janvier-europe.com) were aged 8 months, ex-breeders, and caged singly at study onset. Mice were maintained on a reversed 12:12 h light-dark cycle (lights off 07:00-19:00 h) in an individually-ventilated caging system (IVC) at 20-22 °C and 50-60% humidity. Cages were type 2L and contained woodchips, a sleep igloo and tissue bedding. Complete-pellet diet (Provimi, Kliba Ltd, Kaiseraugst, Switzerland) and water were available continuously and *ad libitum*. In the week prior to CSD, all C57BL/6 mice were handled and weighed on five days; body weights were used to assign mice to CSD and control (CON) groups such that mean weight per group was counter-balanced.

The study was conducted under a permit (110/2009) for animal experimentation issued by the Veterinary Office, Zurich, Switzerland. All efforts were made to minimize the number of mice used and any unnecessary stress to those mice that were used, including refinement of the published protocol for CSD (Golden et al., 2011).

## 3.3 Chronic social defeat

Social defeat sessions were conducted under dim lighting on 15 consecutive days (versus 10 days in the standard protocol). On the day prior to day 1 of CSD, a CD-1 mouse demonstrated to be aggressive (Golden et al., 2011) was placed in one compartment of a cage (Indulab, Gams,

Switzerland, www.indulab.ch) containing a longitudinal divider made from transparent Plexiglas and perforated with multiple holes (∅ = 10 mm) for sensory interactions. To prevent bite wounds, a major concern in the standard protocol (Golden et al., 2011), the lower incisors of these CD-1 mice were trimmed using rodent tooth-cutting forceps (Precision Surgical International, USA, www.psint.net) under brief isoflurane anasethesia; this was repeated at 3-day intervals across CSD. Daily CSD was conducted between 14:00-17:00 h. On day 1, each CSD C57BL/6 mouse was removed from its home cage, weighed and placed in the compartment of a CD-1 mouse. Behaviour was observed and the duration of each physical attack was timed; mice remained together until either a cumulative total of 60-sec physical attack had occurred or 10 min had elapsed. The CSD mouse was controlled for the absence of wounding; skin abrasions did occur rarely but skin penetration never occurred. The CD-1 mouse was removed and weighed. The CSD mouse remained in the compartment in which it had been attacked and the CD-1 mouse was placed in the opposite compartment, allowing continuous olfactory, visual and auditory contact during the following 24 h. Because of the need for careful behavioural observation and timing of attacks, CSD was conducted with only two cages at a time, in contrast to 10 cages at a time in the standard protocol (Golden et al., 2011). On day 2, the CSD - CD-1 mice pairings were rotated so that each CSD mouse was confronted with a novel CD-1 mouse and *vice versa*. The procedure was continued until day 15. Thereafter, CSD mice remained caged next to the same CD-1 mice and there were no further attack sessions or rotations. Control (CON) mice remained in littermate pairs, the standard caging condition in our laboratory, and were handled and weighed daily. In the standard protocol, pairs of CON mice are caged singly separated by dividers (Golden et al., 2011). Comparison of CON mice pairs maintained together or separated with a divider demonstrated no significant effect on behaviour in contextual fear conditioning or two-way active escape-avoidance (unpublished data).

## 3.4 Motor activity, contextual fear conditioning and aversive stimulus control

Behavioural tests were conducted under dim lighting in a room adjacent to the mouse holding room, between 08:30-12:00 h. Motor activity, contextual fear conditioning and two-way active escape-avoidance tests were all conducted using a single multi-purpose system co-developed with the manufacturer (Multi Conditioning System, TSE Systems GmbH, Bad-Homburg, Germany), details of which are given in Pryce et al. (2012).

**Motor activity**

The mouse was placed on the grid floor in the empty arena for 15 min without presentation of any further stimuli. Distance moved (arbitrary units/min, a.u./min) and % time spent freezing in the horizontal and vertical planes were recorded continuously. When the mouse did not make any movement for a period of at least 2 sec this was recorded by the system as freezing. Mean distance moved/min and % time spent freezing were calculated. This test was conducted in Experiment (Expt) A at CSD day 14 and in Expt B at days -1, 7, 13 and 30. In Expt B, faecal boli were collected from the waste tray after each test for measurement of basal concentrations of the major faecal corticosterone (CORT) metabolite (see Corticosterone measurement).

**Contextual fear conditioning**

The mouse was placed on the grid floor in the empty arena and challenged with 15 inescapable electroshocks each of 0.15 mA x 3 sec and delivered at inter-electroshock intervals (ITI) of 50 sec. Mean distance moved during the electroshocks and mean % time spent freezing during ITIs were calculated. This test was conducted in Expt A at day 15 and in Expt B at day 31.

**Two-way active escape-avoidance test/Aversive stimulus control**

The mouse was placed on the grid floor in the arena which contained a central divider with an opening ("gate") through which it could transfer from one side of the arena to the other. A first stage consisted of 10 trials in which a 12-sec tone (5 kHz, 85 dB, conditioned stimulus, CS) preceded an inescapable electroshock (0.15 mA x 3 sec) with inter-stimulus intervals (ITI) of 50 sec. Immediately thereafter, mice were challenged with 30 trials in which the 10-sec CS now preceded an escapable electroshock (0.15 mA x 5 sec maximum) with ITI of 50 sec. If the mouse did not transfer from one side of the arena to the other during the CS+electroshock, this was scored as an escape failure; if the mouse transferred during the electroshock this was scored as an escape response; if the mouse transferred during the CS this was scored as an avoid response. The CS-inescapable electroshock stage was used to establish the CS as an aversive stimulus and to ensure that all mice were exposed to it equally prior to the onset of testing of active escape-avoidance. The following measures were calculated for two-way active escape-avoidance: mean motor activity during electroshock, mean % time freezing during ITI and CS, total escape failures, total escapes, total avoids, and escape-avoid latency. This test was conducted in Expt A at day 16 and in Expt B at day 32.

## 3.5 Treadmill fatigue test/one-way active escape-avoidance

To assess fatigue, mice were tested on a mouse single lane treadmill (Panlab/Harvard Apparatus, Cornellà, Spain) inclined at 30° and with an electroshock grid at its lower end. By running up-hill, mice could avoid the electroshock grid or, if they had failed to run and travelled back onto the grid, escape from it. A pilot experiment in non-manipulated mice demonstrated that a treadmill speed of 20 cm/sec was a moderate-running speed, and that 25 cm/sec was a fast-running speed that mice could maintain for at least 20 min. The habituation-day session consisted of 2 min at 0 cm/sec, 5 min at 15-20 cm/sec at 1 min increments, and 5 min at 20 cm/sec. Total number and duration of electroshocks were scored automatically. On test days, the schedule consisted of 2 min at 0 cm/sec, 5 min at 20 cm/sec, and 20 min at 25 cm/sec i.e. test running speed. Measures, scored automatically, were test duration at 25 cm/sec (maximum 20 min) and total electroshock duration (maximum 10 sec), i.e. the session was terminated prior to 20 min if subjects accumulated a total of 10 sec electroshock. This test was conducted in Expt B at days 17-18 and 29.

## 3.6 Hot plate test

To assess pain sensitivity, a hot plate test was conducted using a programmable thermoelectric heating plate (Teca, Chicago IL, USA) set at 50 °C, with a transparent Plexiglas chamber placed onto the plate (Pryce et al., 2012). Hot plate tests were conducted at 16:00-17:00 h. The mouse was removed from the home cage and placed inside the chamber. The latency (sec) from the onset of the test until the first occurrence of one of the following behaviours was scored: licking a forepaw, licking a

hind paw, lifting a hind paw, jumping. The mouse was then immediately removed from the hot plate. The maximum test duration was 60 s and 60 s was the latency score given to mice that did not exhibit one of the target behaviours. This test was conducted in Expt A at day 17.

## 3.7 Water consumption test and saccharin preference test

For the water consumption test, water was presented in a 15 ml Falcon™ tube (BD, Franklin Lakes, USA). The tube tip was cut off and the filled tube was weighed and placed on the cage grid directly after CSD or CON-handling (14:00-17:00 h). After 24 h the tubes were re-weighed to give daily water consumption (1 g = 1 ml). For CON mice, consumption was divided by 2 to give the average per mouse and only one value per CON pair was taken for statistical analysis. This test was conducted daily in Expt B at CSD days 1 to 10.

For the saccharin preference test, the same modified tubes as for the water consumption test were used. Depending on test day, the saccharin (saccharin sodium salt hydrate, Sigma-Aldrich, St. Louis, USA) concentration used was 0.15 % or 0.5 % in water (w/v). One saccharin and one water tube were placed adjacently on the cage grid from 08:00 to 16:00 h (CSD session started at 16:15 h on this day). Percent saccharin preference was calculated as (Δ saccharin mass/(Δ saccharin mass + Δ water mass)) x 100. The left-right location of the two tubes was counter-balanced within CSD and CON groups. This test was conducted in Expt B at CSD days11 and 16.

## 3.8 Blood, brain, spleen and adrenal collection

For blood factor (Expt A) and fresh-fixed brain (Expt C) studies, mice were decapitated and trunk blood was collected in EDTA-coated tubes (Microvette 500 K3E, Sarstedt) and placed on ice. The brain was removed, rinsed with ice-cold saline, frozen on dry ice and stored at -80° C. Bloods were centrifuged at 3000 rpm and 4° C for 10 min, plasma aliquots transferred to cryotubes (Protein LoBind, Eppendorf) and stored at -80° C. Adrenal glands and spleen were removed, cleaned of fat and connective tissue and weighed.

## 3.9 Plasma cytokine measurement

Plasma titres of the pro-inflammatory cytokines TNF and IL-6 were measured using a multiplexed particle-based flow cytometric cytokine assay (Marques-Vidal et al., 2011). Lower limits of detection for TNF and IL-6 were 0.5 pg/mL.

## 3.10 Corticosterone measurement

Plasma CORT concentrations were measured using an EIA kit (AssayMax CORT ELISA kit; AssayPro, Saint Charles, MO, USA). Plasma was diluted 1:50 in ELISA diluent and heated at 90° C for 10 min for transcortin denaturation (Pryce et al., 2001). All further steps were performed according to the manufacturer's protocol. Faecal samples were dried and powdered, 20-50 mg were extracted in 80% methanol (Palme et al., 2013). After centrifugation supernatants were stored at -20°C and transferred to Vienna. Faecal CORT metabolites were measured using an in-house 5α-pregnane-3ß,11ß,21-triol-20-one EIA (Touma et al., 2003), to provide a non-invasive correlate of basal concentrations of blood CORT.

## 3.11 Brain region-specific transcriptome expression

Frozen brains were sectioned coronally at 1 mm intervals using a stainless-steel brain matrix (model MMCS-1, Plastics One) and single-edge blades (model 10-100-063, Apollo Herkenrath, Solingen, Germany). Regions of interest (ROIs), namely ventral HIPP (vHIPP), AMYG and medial PFC (mPFC; infralimbic cortex + ventral prelimbic cortex) were micro-dissected bilaterally from the corresponding sections using a brain punch (∅ = 1 mm, model 57397, Stoelting Europe) and a mouse brain atlas (Franklin and Paxinos, 2008): mPFC (1 biopsy/hemisphere) at Bregma 2.1 to 1.2 ± 0.2 mm; AMYG (1 biopsy/hemisphere) at Bregma -0.7 to -1.7 ± 0.3 mm; vHIPP (2 biopsies/hemisphere) at Bregma -2.8 to -3.9 ± 0.3 mm. Tissue mass was 0.6-0.8 mg per biopsy. All microdissection steps were conducted at -18 °C. Brain biopsies were stored in 1.5 ml Protein LoBind polypropylene tubes (Eppendorf, model 0030 108.116) at -80 °C.

For RNA isolation, samples were homogenized in 400 µl 1%-ß-mercaptoethanol-RLT-buffer, and total RNA was isolated from 200 µl homogenate using the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The high quality of the RNA was determined using the RNA 6000 Nano kit and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Preparation of the mRNA sequencing library for each sample was conducted using 200 ng of total RNA in the TrueSeq RNA Sample Preparation Kit v2-Set B (RS-122-2002, Illumina Inc, San Diego, USA); this yielded fragments of an average size of 275 bp including the adapters with their library-specific indices supplied by the manufacturer. Concentration of the libraries was normalized and eight libraries were multiplexed and clustered on the cBot Instrument (Illumina Inc) using the TruSeq SR Cluster Kit v3 - cBot – HS (GD-401-3001, Illumina Inc). Sequencing was performed with single reads of 52 bp using the TruSeq SBS Kit HS - v3 (FC-401-3002, Illumina Inc) on an Illumina HiSeq2000 instrument. Read alignment of the RNA-seq dataset was performed using STAR aligner software (Dobin et al., 2013). Gene expression analysis was conducted using Cufflinks software with differential expression of genes in CSD versus CON mice identified using the Cuffdiff 2 test (Trapnell et al., 2013). Briefly, the Cuffdiff 2 test assesses between-group log-fold change in gene expression against the null hypothesis of no change whilst controlling for false discovery rate (FDR) using the Benjamini-Hochberg correction for multiple-testing. The criteria used to identify CSD-CON differential gene expression were reads per kilo base per million (RPKM) > 5, fold change > 1.4, and $q < 0.05$. Within and between the three regions of interest, those genes that met these criteria for significantly increased or decreased differential expression in CSD versus CON mice were investigated in terms of canonical pathways and upstream regulator networks, using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, USA).

## 3.12 Statistical analysis

Statistical analysis of CSD effects on behaviour and physiology was conducted using SPSS (version 20, SPSS Inc., Chicago IL, USA). In most cases ANOVA was used, with a between-subject factor of group (CSD, CON) and, depending on parameter, a within-subject factor of CSD day block (e.g. body weight (BW), water consumption). *Post hoc* testing was conducted using the Bonferroni correction for multiple comparisons. In cases where there was a non-normal distribution of data i.e. IL-6 plasma

concentrations, the Mann-Whitney *U* test was used. Statistical significance was set at $p < 0.05$. Where an estimate of variance is given this is the standard deviation (SD).

# 4.RESULTS

## 4.1 Repeated measures of physical and behavioural status

The mean duration of CSD attacks per C57BL/6 mouse was 47±3, 44±4 and 46±5 sec in experiments A, B and C, respectively. Body weight (BW) was taken daily: there was no effect of CSD on absolute BW (Fig. 2A), whereas day-to-day BW delta (ΔBW) was increased in CSD relative to CON mice ($F(1, 25) = 8.0$, $p < 0.009$): this was the case at CSD days 2-6 and 7-11 (Fig. 2B and Fig. S1A, S1B and S1E, S1F). For basal motor activity in a neutral environment, there was a significant group x time-block interaction ($F(2, 42) = 5.8$, $p < 0.006$): *post hoc* analysis indicated no CSD effect at day -1 and significantly decreased motor activity in CSD versus CON mice at days 7 and 13 (Fig. 2D). Water consumption measured at days 2-6 and 6-10 was increased in CSD mice ($F(1, 16) = 7.5$, $p < 0.02$; Fig. 2C). For saccharin preference in a two-bottle test conducted at days 11 and 16, there was no CSD effect ($p = 0.07$): preference score for CSD mice was 95.9±3.0 % and that of CON mice 97.9±1.5 % (Fig. S1C). Basal concentrations of the major faecal CORT metabolite, measured as a non-invasive biomarker of plasma CORT levels, did not differ between CSD and CON mice in faecal samples collected at days -1, 7 and 13 ($p = 0.78$, Fig. S1D).

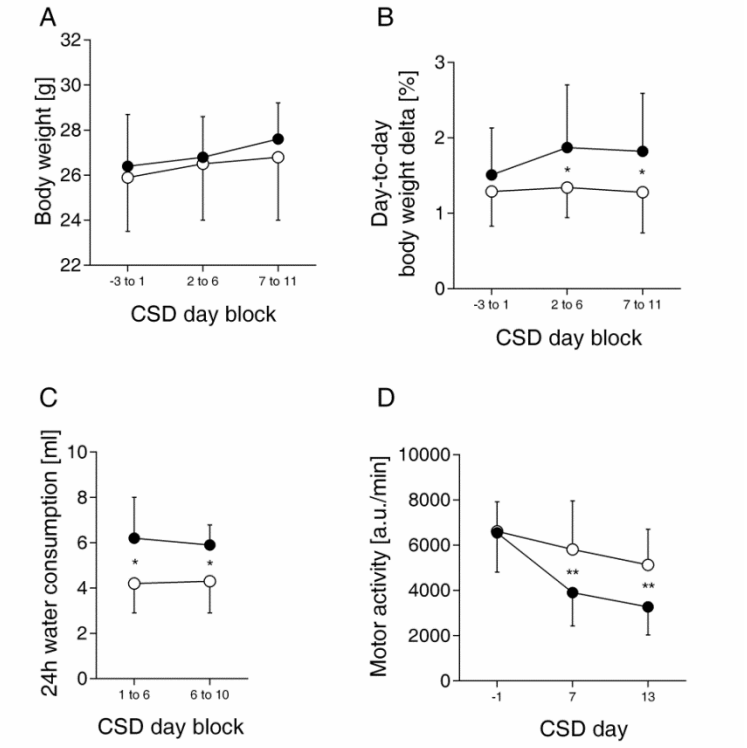



Figure 2: Effects of chronic social defeat on repeated physical and behavioural measures. (A) Absolute body weight at 14:00 h (Expt. A, CON = 13 (open circles), CSD = 14 (filled circles)). (B) Percent day-to-day body weight delta (Expt. A, abs(BW day n – BW day n-1))/(BW day n-1)*100). (C) 24-h water consumption (Expt. B, CON = 10, CSD = 13). (D) Motor activity in neutral arena (Expt. B). Values are mean +/- SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0005$.

## 4.2 Contextual fear conditioning and pain sensitivity

Short-term effects of CSD (Expt A, Fig. 1) were tested in terms of psychomotor activity at day 14 and contextual fear conditioning at day 15. CSD mice exhibited decreased psychomotor activity: CSD 3961±1489 a.u./min, CON 6093±2272 a.u./min ($t$ = 2.905, $df$ = 25, $p$ < 0.008). In the same test, CSD and CON mice spent a similar and low % time freezing ($p$ = 0.55, Fig 3A). In the test of contextual fear conditioning, CSD mice exhibited an increased % time freezing relative to CON mice ($t$ = -4.914, $df$ = 25, $p$ < 0.0005, Fig 3A). In the study of long-term effects of CSD (Expt B, Fig. 1), there was no effect of CSD on activity in the psychomotor motor activity test at day 30: CSD 3381±1625 a.u./min, CON 4682±1998 a.u./min ($p$ = 0.10). In the same test, CSD and CON mice spent a similar and low % time freezing ($p$ = 0.06, Fig. 3B). In the contextual fear conditioning test at day 31, CSD mice exhibited an increased % time freezing relative to CON mice ($t$ = -4.022, $df$ = 21, $p$ < 0.001, Fig 3B).

In terms of pain sensitivity measures, there was no short-term effect of CSD on motor reactivity to the electroshock in the contextual fear conditioning test ($p$ = 0.13, Fig. 3C), and also no effect in the hot plate test at day 17 ($t$ = 1.356, $df$ = 25, $p$ = 0.19, Fig. 3D). In the study of long-term effects, in addition to the increased contextual freezing at day 31, CSD mice exhibited decreased motor reactivity to the electroshock ($t$ = 4.272, $df$ = 21, $p$ < 0.0005, Fig. 3E).

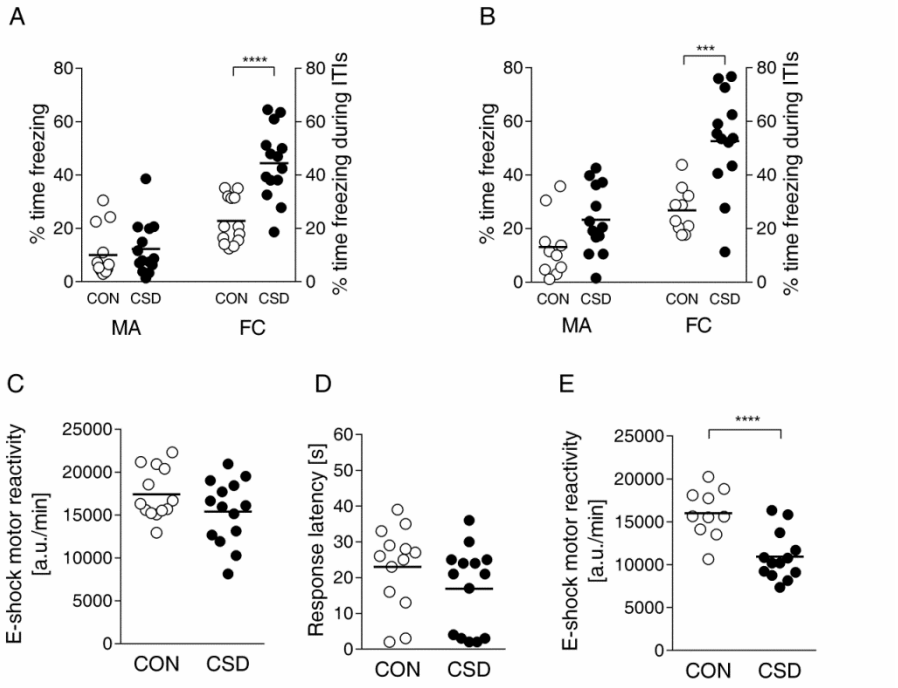



Figure 3: Short- and long-term effects of chronic social defeat on baseline and conditioned freezing. (A) Short-term (Expt. A, CON = 13 (open circles), CSD = 14 (filled circles)) CSD effect on % time freezing in the motor activity test (MA, day 14) and during inter-trial intervals (ITIs) in the contextual fear conditioning test (FC, day 15). (B) Long-term (Expt. B, CON = 10 (open circles), CSD = 13 (filled circles)) CSD effect on % time freezing in MA (day 30) and ITIs of FC (day 31). Short-term CSD effects on (C) motor reactivity to electroshock (E-shock) in FC, and (D) pain response latency in the hot plate test (day 17). (E) Long-term CSD effect on motor reactivity to E-shock in FC. * $p$ < 0.05, ** $p$ < 0.01, *** $p$ < 0.001, **** $p$ < 0.0005.

### 4.3 Two-way active escape-avoidance/Aversive stimulus control

On the day following contextual fear conditioning, a two-way active avoidance test was conducted. The arena was the same as for contextual fear conditioning with the addition of a central divider and gate. The electroshock (5 sec) was now escapable by transferring through the gate; it was preceded by a tone CS (10 sec), and transferring through the gate during the CS terminated it and resulted in avoidance of the electroshock. Measures were % time freezing during ITIs and CSs, avoids i.e. compartment transfer during the CS, electroshock escapes, avoid-escape failures, avoid-escape latency (maximum 15 sec), and motor reactivity to electroshock. In the study of short-term effects, at day 16 CSD mice exhibited increased % time freezing relative to CON mice during ITIs ($t$ = -4.738, $df$ = 24, $p < 0.0005$, Fig. 4A) and during CSs ($t$ = -3.728, $df$ = 24, $p < 0.001$, Fig 4A). There was no significant effect of CSD on avoid-escape failures ($p$ = 0.38, Fig. 4B) or avoids ($p$ = 0.14, Fig. 4C). When mice were categorized according to whether they made ≤ 4 or ≥ 5 avoids, a higher proportion of CSD than CON mice exhibited ≤ 4 avoids ($\chi^2$ (1, $N$=27) = 4.74, $p < 0.03$, Fig. 4D). CSD and CON mice exhibited similar motor reactivity to the electroshock ($p$ = 1.00, Fig. S2A). In the study of long-term effects, at day 32 there was no effect of CSD on % time freezing during either ITIs ($p$ = 0.62, Fig 4E) or CSs ($p$ = 0.22, Fig 4E). CSD mice exhibited more avoid-escape failures than did CON mice ($t$ = -3.168, $df$ = 21, $p < 0.005$, Fig. 4F). There was no significant effect of CSD on avoids ($t$ = 1.393, $df$ = 21, $p < 0.178$, Fig. 4G), but CSD mice exhibited less escapes than CON mice ($t$ = 2.999, $df$ = 21, $p < 0.007$, Fig. 4H). CSD mice exhibited decreased motor reactivity to the electroshock ($t$ = 3.640, $df$ = 21, $p < 0.002$, Fig. S2B). The avoid-escape latency was significantly greater in CSD than CON mice ($t$ = -2.661, $df$ = 21, $p < 0.015$, Fig. S2C).

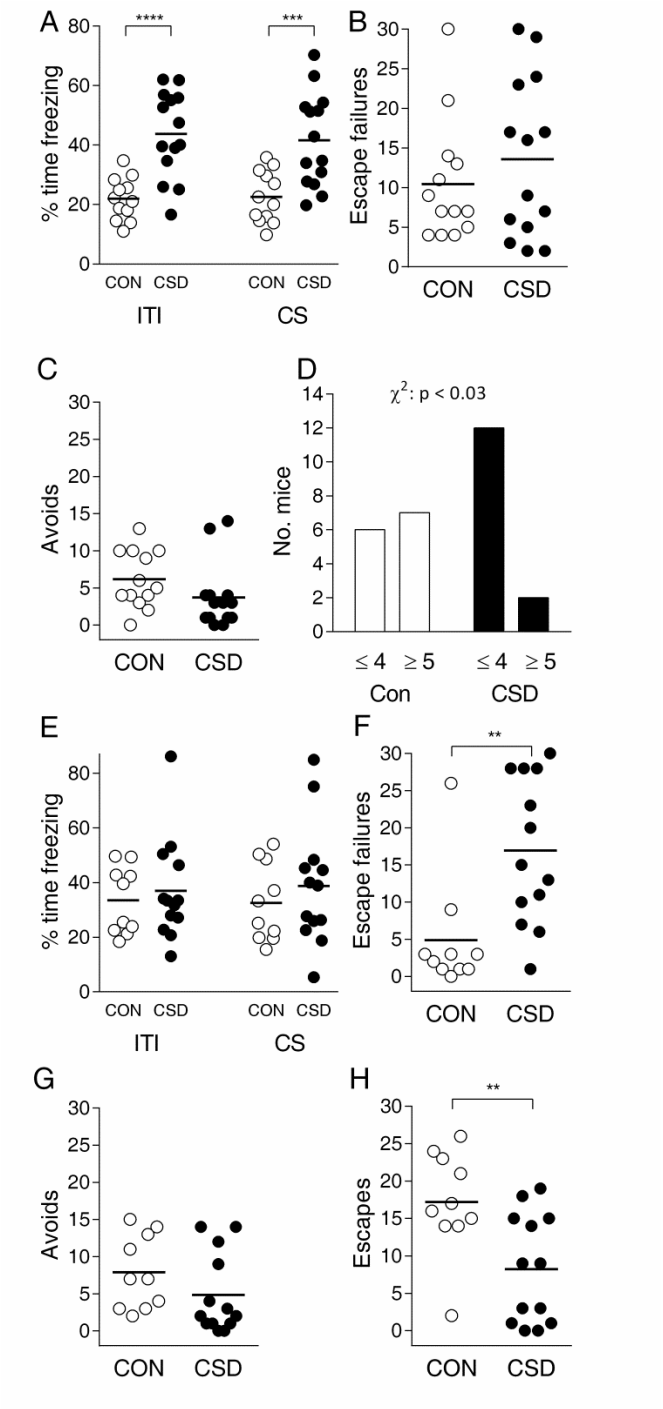



Figure 4 : Short- and long-term effects of chronic social defeat in two-way active escape-avoidance test (aversive stimulus control). Short-term (Expt A, CON = 13 (open circles), CSD = 14 (filled circles)): (A) Mean per mouse % time freezing during inter-trial intervals (ITI) and conditioned stimulus presentations (CS, 10-s tone). (B) Total escape failures. (C) Total avoid responses. (D) Frequency distribution of avoid responses using categories of ≤4 or ≥5 avoids. Long-term (Expt B, CON = 10 (open circles), CSD = 13 (filled circles)): (E) Mean % time freezing during ITI and CS. (F) Total escape failures. (G) Total avoid responses. (H) Total escape responses. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0005$.

## 4.4 Treadmill fatigue

The short- and long-term effects of CSD on treadmill behaviour were assessed. At treadmill habituation on day 17, there was no effect of CSD on total duration of electroshock received ($t$ = -1.180, $df$ = 19, $p$ = 0.25, Fig. 5A). At treadmill test on day 18, test running time achieved was decreased in CSD mice relative to CON mice ($t$ = 4.900, $df$ = 21, $p$ < 0.0005, Fig. 5B) and total duration of electroshock received was significantly increased ($t$ = -3.631, $df$ = 21, $p$ < 0.002, Fig. 5C). These CSD effects were observed again in the same mice in the treadmill test at day 29: test running time achieved was reduced ($t$ = 6.058, $df$ = 21, $p$ < 0.0005, Fig. 5D) and total electroshock duration increased ($t$ = -7.420, $df$ = 21, $p$ < 0.0005, Fig. 5E), in CSD relative to CON mice.

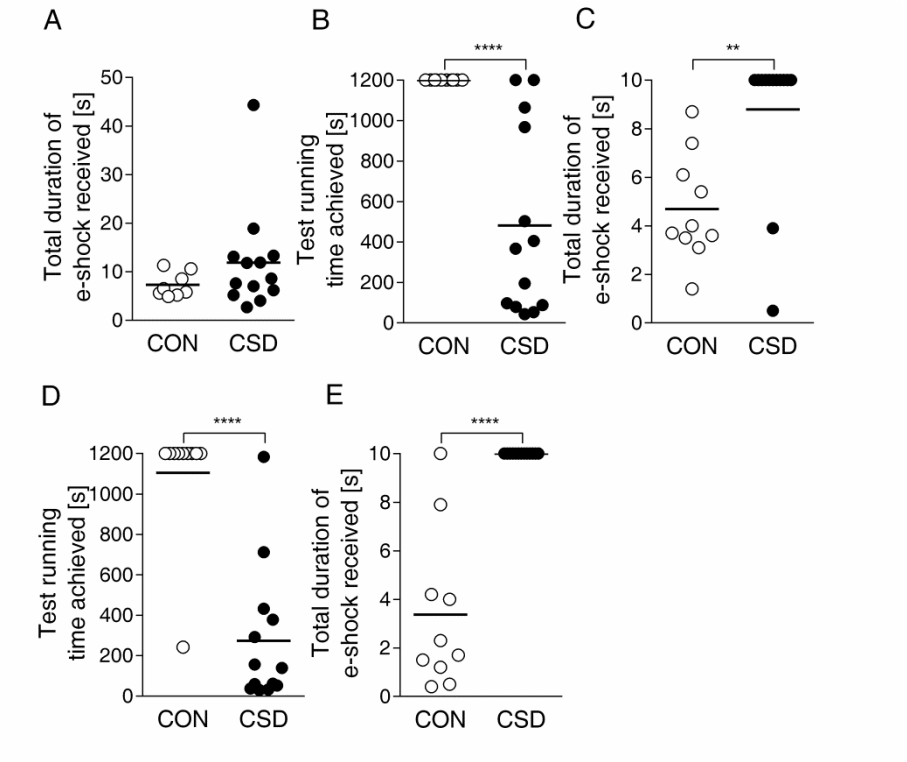



Figure 5: Short- and long-term effects of chronic social defeat in treadmill fatigue test (all data from Expt. B, CON = 10 (open circles), CSD = 13 (filled circles)). Short-term: (A) Total duration of electroshock (E-shock) received during habituation (day 17), (B) Test running time achieved (day 18), (C) Total duration of E-shock received at test (day 18). Long-term: (D) Test running time achieved (day 29), (E) Total duration of E-shock received at test (day 29). * $p$ < 0.05, ** $p$ < 0.01, *** $p$ < 0.001, **** $p$ < 0.0005.

## 4.5 Physiological status

In the study of short-term effects, mice were euthanized at day 18. CSD led to an increase in plasma basal concentrations of TNF ($t$ = -3.099, $df$ = 25, $p$ < 0.005, Fig. 6A) and IL-6 ($U$ = 45.5, $N$ = (13, 14), $p$ < 0.03, Fig. 6B). Relative to CON mice, CSD mice exhibited increased spleen weight, both absolute ($t$ = -4.26, $df$ = 25, $p$ < 0.0005, Fig S3A) and relative to BW ($t$ = -3.66, $df$ = 25, $p$ < 0.001, Fig. 6C). There was no effect of CSD on plasma basal concentration of CORT ($p$ = 0.60, Fig. 6D). Nonetheless, relative to CON mice, CSD mice exhibited increased total adrenal gland weight, both absolute ($t$ = -

2.76, *df* = 25, *p* < 0.01, Fig S3B) and relative to BW (*t* = -2.57, *df* = 25, *p* < 0.02, Fig. 6E). In the separate cohort of mice euthanized at day 17 for gene expression analysis, CSD induction of splenomegaly and adrenomegaly was again observed (Fig. S3C and D).

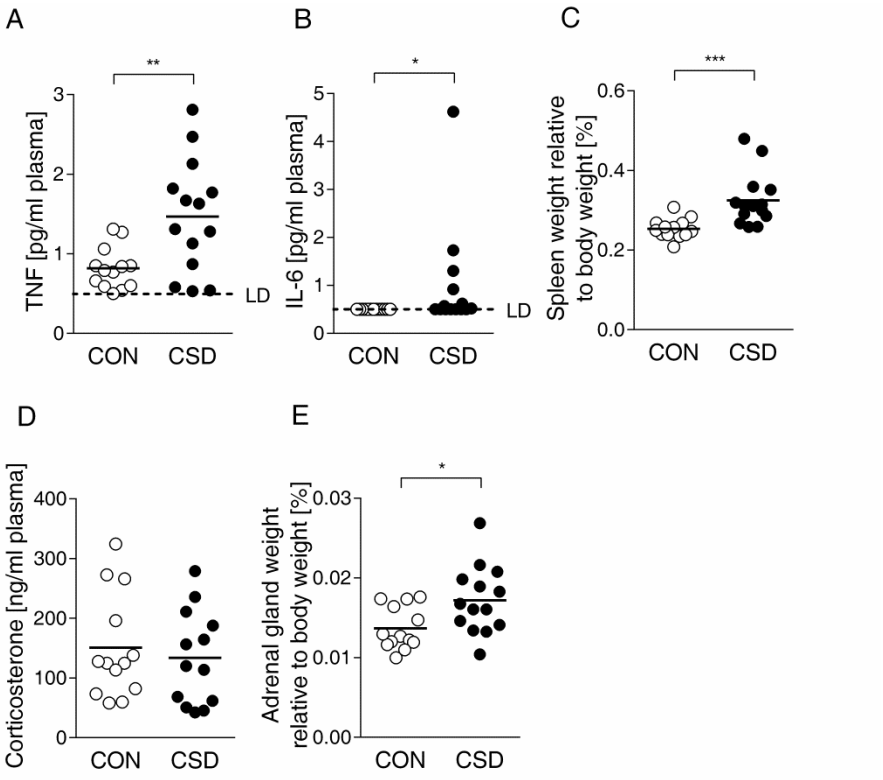



Figure 6: Short-term effects of chronic social defeat on physiological measures (Expt. A, CON = 13 (open circles), CSD = 14 (filled circles)). (A) Blood plasma concentration of tumor necrosis factor (TNF). (B) Blood plasma concentration of interleukin-6 (IL-6). (C) Spleen weight relative to body weight. (D) Blood plasma concentration of corticosterone. (E) Adrenal gland weight relative to body weight. * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001, **** *p* < 0.0005.

## 4.6 Brain region-specific transcriptome expression status

Mice were euthanized at day 17 and differential gene expression was investigated in vHIPP, AMYG and mPFC using next generation sequencing. In vHIPP, CSD mice exhibited significantly increased and decreased expression of 21 and 33 genes, respectively (Fig. 7, Tables S3 and S4). There was no canonical pathway that was enriched by more than one of these differentially expressed genes. Upstream analysis identified that each of the pro-inflammatory cytokines TNF, IL-6 and IL-3 are regulators of the CSD-induced differential gene expression; the specific cytokine-gene relationships are given in Table S1. In AMYG, CSD mice exhibited increased and decreased expression of 22 and 48 genes, respectively (Fig. 7, Tables S3 and S4). In terms of canonical pathways, these genes were primarily associated with T cell receptor signalling (4 genes: *Cd4* (↓)*, Jun* (↑)*, Ptprc* (↑)*, Shb* (↓)), G-protein coupled receptor signalling (5 down-regulated (↓) genes: *Drd2, Adora2a, Chrm3, Htr2a, Pde10e*), and CCR5 signalling in macrophages (3 genes: *Cd4* (↓)*, Jun* (↑)*, Gng7* (↓)). A number of the genes exhibiting altered expression are dopamine (DA) binding and involved in either mediating or

regulating DA neurotransmission (Table 1). One of the de-regulated DA-binding genes, *Slc29a4* (↑), also binds serotonin (5-HT), and the gene for the 5-HT receptor Htr2a was also de-regulated (↓). Furthermore, upstream analysis identified DA (Table 1), as well as cocaine and BDNF, as significant regulators of the genes exhibiting CSD-induced differential expression in AMYG. In mPFC, CSD mice exhibited increased and decreased expression of 6 and 14 genes, respectively (Fig. 7, Tables S3 and S4). As for AMYG, the canonical pathways enriched by these genes had immuno-inflammatory function, namely IL-12 signalling and production in macrophages, and production of nitric oxide and reactive oxygen species in macrophages (same 3 genes for both pathways: *Apod* (↓), *Fos* (↑), *Prkcd* (↑)) (Fig. 7). Upstream analysis identified the NMDA receptor complex as the major regulator of the genes that exhibited CSD-induced differential expression in mPFC: this receptor is associated with up-regulation of each of the genes *Arc*, *Fos* and *Penk*, which exhibited increased expression in CSD mice (Table S2). Upstream analysis also identified the NMDA antagonist ketamine as a significant up-regulator of *Arc* and *Fos.*

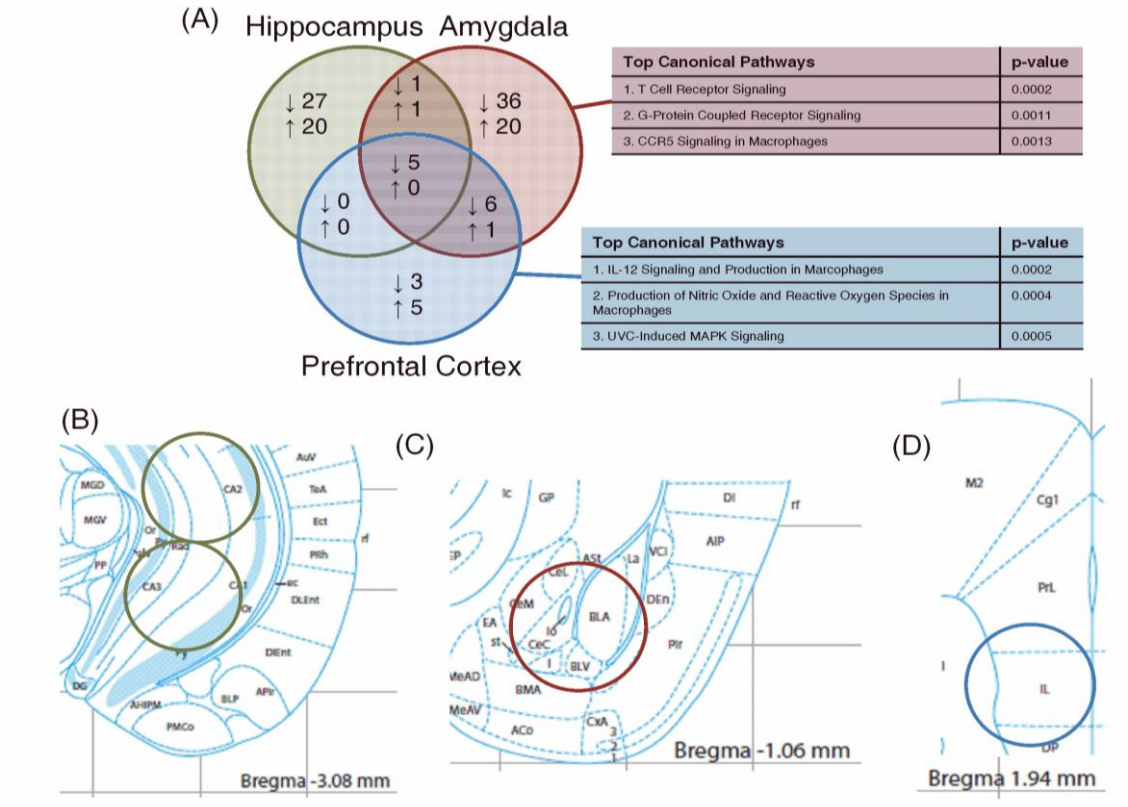



Figure 7: Short-term effects of chronic social defeat on gene expression assessed using next generation sequencing (Expt C, CON = 12, CSD = 12). (A) Venn-diagram of down- (↓) and up-regulated (↑) genes in ventral hippocampus, amygdala and medial prefrontal cortex, and the corresponding top canonical pathways identified using Ingenuity Pathway Analysis. Representative figures (from (Franklin and Paxinos, 2008)) depicting the region of analysis for (B) ventral hippocampus, (C) amygdala and (D) medial prefrontal cortex.

**Table 1. Genes exhibiting altered expression in CSD versus control mice in amygdala that regulate and/or are regulated by dopamine neurotransmission**

| Gene | | Up-/down-regulation | Fold change[1] | Regulator of DA function | Regulated by DA function[2] | Reference |
|---|---|---|---|---|---|---|
| *Adora2a* | Adenosine A2a receptor | ↓ | 1.9 | + | | |
| *Darpp-32* | Dopamine and cAMP regulated phosphoprotein 32 | ↓ | 1.7 | + | + | Li et al. (2013) |
| *Drd2* | Dopamine receptor D2 | ↓ | 1.8 | + | | Boison et al. (2012) |
| *Gabrd* | Gamma-aminobutyric acid (GABA) A receptor, delta | ↓ | 1.6 | | + | |
| *Gng7* | Guanine nucleotide binding protein, gamma 7 | ↓ | 1.4 | + | | Sasaki et al. (2013) |
| *Gpr88* | G protein-coupled receptor 88 | ↓ | 1.8 | + | | Logue et al. (2009) |
| *Kcnk2* | Potassium channel, subfamily K, member 2 | ↓ | 1.5 | | + | |
| *Pde10a* | Phosphodiesterase 10A | ↓ | 1.5 | | + | |
| *Rgs9* | Regulator of G-protein signaling 9 | ↓ | 2.0 | + | | Celver et al. ( 2012) |
| *Tac1* | Tachykinin, precursor 1 | ↓ | 1.9 | | + | |
| *Gpr68* | G protein-coupled receptor 68 | ↑ | 1.4 | | + | |
| *Jun* | Jun proto-oncogene | ↑ | 1.4 | | + | |
| *Slc29a4* | Solute carrier family 29 (nucleoside transporters), member 4 | ↑ | 1.4 | + | | Engel et al. ( 2004) |

[1] q-values for fold change = 0.003 in all cases

[2] upstream analysis conducted using Ingenuity Pathway Analysis

# 5.DISCUSSION

In C57BL/6 mice, chronic exposure to uncontrollable psychosocial stress, an environmental manipulation with aetiological validity for depression, induced robust short- and long-term behavioural effects in the form of increased fear reactivity to a context associated with an aversive stimulus, decreased operant control of an aversive stimulus, and increased fatigue when required to exert effort to avoid an aversive stimulus. These CSD-induced behavioural states have face validity for some of the core psychopathologies of depression. In addition, they are relevant to the generalised helplessness theory of depression onset and maintenance, given that the experience of social defeat was generalised into altered emotional, motivational and cognitive responses to a different category of unconditioned aversive event, namely electroshock. Given its aetiological and face validity, it can be deduced that this novel depression model will also possess construct validity, i.e. to depression aetio-pathophysiology. Accordingly, it could well be relevant to depression and to next-generation anti-depressant neuropsychopharmacology, that CSD, in mice studied 2-3 days after the end of the stressor, induced: immuno-inflammation markers in terms of increased blood titres of TNF and IL-6 and splenomegaly, in line with the hyper-activity of these systems in depression; cortico-limbic de-regulation of expression levels of genes important in immuno-inflammation pathways; and cortico-limbic de-regulation of expression levels of genes important in DA, adenosine, 5-HT and glutamate neurotransmission.

The insightful first development of the CSD manipulation used C57BL/6 mice as both aggressors and subjects and was carried out for 20 days (Kudryavtseva et al., 1991). The authors emphasized the aetiological validity of CSD for depression given that it primarily comprised chronic psychosocial emotional stress i.e. the chronic distal sensory stimuli associated with acute physical attacks were important rather than the attacks *per se* (Kudryavtseva et al., 1991). Their detailed observations revealed that daily introduction of a novel aggressor added unpredictability, and that the repeated displays of submissive behaviour were ineffective in reducing attacks such that defeat was experienced repeatedly, i.e. CSD mice experience stressor uncontrollability (Kudryavtseva et al., 1991; Pryce et al., 2011). Subsequent modifications were to use ex-breeder CD-1 mice as aggressors and to reduce the duration from 20 to 10 days (Golden et al., 2011). In the present study, 15-day CSD was used in combination with two refinements aimed at preventing bite wounds, namely trimming of the incisor teeth of CD-1 mice and timing of attacks to ensure that they had a maximum duration of 60 sec/day. The absence of physical injury that resulted from these refinements, together with the observation that submissive behaviour did not control aggression received, further added to the certainty that a psychosocial emotional stressor with aetiological validity had been achieved.

The original behavioural effects described for CSD were decreased activity in open field and forced swim tests, consistent with psychomotor retardation (Avgustinovich et al., 2005; Haque et al., 2012). Reward sensitivity, as measured using the sucrose consumption or preference tests, was decreased in CSD mice (Haque et al., 2012; Venzala et al., 2013). In the elevated plus maze, an approach-avoidance conflict anxiety test, CSD has an anxiogenic effect (Avgustinovich et al., 2005; Haque et al., 2012; Krishnan et al., 2007; Venzala et al., 2013). In fear conditioned freezing, CSD

induced increased expression of fear to context and CS (Yu et al., 2011). The same study reported that CSD impaired spatial working memory in a T-maze test. A major focus for the study of behavioural effects of CSD has been social avoidance, measured using the partition test and the social approach-avoidance test. In the former, relative to CON mice, CSD mice spend less time near the partition that separates them from a novel mouse of the same strain (Avgustinovich et al., 2005). The latter test consists of two sessions in an arena containing a small wire-mesh cage: in the first session, the subject mouse explores the arena with empty cage and in the second session with the cage containing a novel CD-1 mouse; CSD mice exhibit decreased time in proximity of the cage in the second session i.e. high social avoidance. Some studies report that CSD leads to a consistent increase in social avoidance relative to CON mice (Berton et al., 2006; Haque et al., 2012); other studies report that approximately half of the CSD mice exhibit high social avoidance ("susceptible") whereas the other half resemble CON mice ("unsusceptible"/"resilient") (Krishnan et al., 2007). It is noteworthy that such social avoidance tests use the same aversive stimulus for both CSD and readout test; that is, a stimulus-specific CSD effect is being demonstrated, and high avoidance of the stimulus experienced as aversive and uncontrollable can even be considered as adaptive behaviour.

In the present study, CSD led to reduced activity in a neutral environment i.e. psychomotor retardation, a common symptom in depression (DSM-5, 2013; ICD-10, 1994). As indicated by the hot plate test, CSD did not change sensitivity to nociceptive pain. When exposed to brief, mild electroshocks in this environment, CSD led to potentiation of contextual fear conditioning, as indicated by increased freezing behaviour, in both the short- and long-term experiments. Potentiated fear conditioning, i.e. increased attribution of emotional valence to stimuli associated with an aversive US, is increased in depression (Nissen et al., 2010). This novel finding that CSD potentiates the acquisition of fear conditioning adds to a previous report that it also increases expression of conditioned fear (Yu et al., 2011). In rat, chronic mild stress led to increased acquisition and expression of contextual fear conditioning (Sandi et al., 2001), and we have also observed that CSD leads to increased acquisition and expression of freezing in CS fear conditioning (unpublished data). Both HIPP and AMYG, and their interaction, are essential to contextual fear conditioning (Maren et al., 2013), suggesting that CSD leads to potentiated aversive stimulus processing in one or both of these regions. Prior to assessing CSD effects on two-way active escape-avoidance, mice underwent CS-inescapable electroshock conditioning to ensure that they all acquired CS aversion prior to the onset of two-way active escape-avoidance testing. In the short-term experiment, relative to CON, CSD led to no effect on escape failure, increased freezing during ITI and CS, and decreased avoidance; in the long-term experiment, CSD led to increased escape failure, decreased electroshock reactivity and no effect on avoidance or freezing. Decreased avoidance and increased escape failure are both indices of decreased control in an aversive environment, so-called helplessness. Using the same apparatus as that used here, repeated exposure of otherwise non-manipulated mice to inescapable electroshocks leads to a subsequent increase in escape failure (associated with decreased electroshock reactivity and no change in freezing) relative to mice that experienced the same electroshocks as escapable, i.e. demonstration of specific learned uncontrollability/helplessness (Pryce et al., 2012). Triangulating the current findings with the evidence that mice are sensitive to (un)controllability leads to the conclusion that, in mice, emotional psychosocial stress induces a state of generalised

uncontrollability/helplessness relative to aversive events. Potential explanations of the findings that the expressions of generalised helplessness differed in the short-term (decreased avoids, increased freezing) and long-term (increased escape failures, decreased electroshock reactivity) include increased accumulation of the neurobiological changes induced by CSD in the long-term experiment, and the methodological difference that treadmill tests preceded the two-way active escape-avoidance test in the long-term experiment. As noted above, generalised learned helplessness (LH) is a major theory for both onset and maintenance of depression (Abramson et al., 1978; Pryce et al., 2011). The proposed mediating processes are increased emotionality (cf. increased freezing in CSD mice), decreased motivation (cf. decreased electroshock reactivity in CSD mice and specific-LH mice (Pryce et al., 2012)), and decreased cognitive expectancy of operant control (cf. decreased avoids or escapes in CSD mice and specific-LH mice). There are rodent and human evidences that (un)controllability, involving integration of emotional and cognitive information, is processed by the prefrontal cortex, specifically prelimbic and infralimbic cortices in rodent and anterior cingulate cortex in human (reviewed in Pryce et al., 2011). Learned helplessness has been associated with increased activity in mPFC inhibitory GABA interneurons, modulated by increased signalling in the 5-HT-protein kinase C (PKC) pathway (Amat et al., 2005). In the specific LH paradigm (Pryce et al., 2012), DA depletion in nucleus accumbens increases specific LH whereas amphetamine reverses it, with both effects mediated primarily via the motivational psychomotor reactivity to electroshock (unpublished data).

Fatigue is a core symptom of depression (DSM-5, 2013; ICD-10, 1994). It is a multi-faceted pathology with components including psychomotor retardation, physical tiredness and mental fatigue (Demyttenaere et al., 2005), and is also likely to contribute to the deficient effortful goal-directed behaviour that is important in depression. Whilst fatigue is a core symptom of depression and although effortful-running fatigue has been studied in mice in the context of exercise physiology, to our knowledge this is the first report of an animal model of psychosocial stress-induced fatigue. The deficit that CSD induced in mouse running to avoid or escape electroshock was specific to the relatively fast treadmill speed, that is, to effortful conditions. Nucleus accumbens DA depletion induces a similar deficit to that of CSD (unpublished data). Whereas the focus of this study was the effects of CSD on processing of aversive events, one test of reward processing was also studied, namely the two-bottle saccharin preference test, and there was no effect of CSD. This is in contrast to previous studies that report decreased sweet-reward preference in CSD mice (see above). One potential explanation for the absence of effect on saccharin preference is that CSD induced polydypsia and the increase in overall fluid intake might have masked any moderate effects on reward sensitivity. Future studies will use operant tests to investigate CSD effects on reward-directed behaviour under effortful conditions e.g. using a progressive ratio schedule of reinforcement (Pryce et al., 2004).

In terms of physiology, CSD did not impact on absolute BW but led to increased day-to-day change in BW. Previous studies report that CSD increases (Bartolomucci et al., 2009), decreases (Kudryavtseva et al., 1991) or has no effect on (Savignac et al., 2011) absolute BW. Both decreased and increased absolute BW are diagnostic symptoms of depression (DSM-5, 2013; ICD-10, 1994); increased BW variability has been associated with high negative affect and low self-esteem (Foreyt et al., 1995; Serdar et al., 2011). Mice that experienced CSD exhibited increased plasma levels of the pro-inflammatory cytokines TNF and IL-6 and splenomegaly, indicating chronic activation of the

peripheral immuno-inflammatory system. Given that the CSD protocol was refined to prevent physical injury, this activation of immuno-inflammatory status can be attributed unequivocally to psychosocial emotional stress. In humans, psychosocial stress causes increased blood levels of pro-inflammatory cytokines and transcription factors (Bierhaus et al., 2003; Kiecolt-Glaser et al., 2005; McDade et al., 2006), and meta-analysis has identified that blood levels of TNF and IL-6 are increased in depression (Dowlati et al., 2010). A previous study in CSD mice also reports that it induces splenomegaly (Bartolomucci et al., 2004). In the present study CSD also led to adrenomegaly, in the absence of changes in basal CORT levels as assessed by plasma total CORT and faecal CORT metabolites. Previous studies of CSD effects on basal plasma CORT report either an increase (Perez-Tejada et al., 2013) or no change (Krishnan et al., 2007); both studies report an increased CORT stress response in CSD mice. In depression, 40-60% of patients exhibit increased basal cortisol levels relative to healthy probands (Parker et al., 2003). The absence of increased basal CORT in the present study indicates that pro-inflammatory activation was not counteracted by increased anti-inflammatory CORT activity in CSD mice (Rhen and Cidlowski, 2005).

Short-term effects of CSD on region of interest gene expression were measured using next-generation sequencing technology in mice that were naïve to behavioural testing, thereby allowing for the study of CSD-induced gene de-regulation *per se*. As described above, the ROIs, namely vHIPP, AMYG and mPFC, are integral to the circuitry underlying the depression-relevant behavioural processes studied here. In vHIPP, CSD led to de-regulation of 54 genes (33 ↓ and 21 ↑); using Ingenuity Pathway Analysis (IPA) upstream analysis, 11 of these genes were identified as having one or more of the pro-inflammatory cytokines TNF, IL-6 and IL-3 among their major regulators; the former two were increased in plasma of a different cohort of CSD mice. The up-regulated genes included the TNF-receptor superfamily gene *Tnfrsf25*, which stimulates NF-κβ and cell apoptosis. In AMYG, CSD led to de-regulation of 70 genes (48 ↓ and 22 ↑). Two of the three top canonical pathways enriched by these genes, as identified by IPA, were T cell receptor signalling and CCR5 signalling in macrophages, again indicating that CSD triggered changes in immuno-inflammation transcription processes in limbic regions of the mouse CNS. In the former pathway, *Ptprc*, an essential regulator of cytokine signalling, was up-regulated in CSD mice; in the latter pathway, *shb*, an inhibitory regulator of T cell receptor, was down-regulated in CSD mice, consistent with disinhibition of T cell signalling (Gustafsson et al., 2011). Another CSD-AMYG down-regulated gene in the CCR5 pathway was *Gng7*: Gng7 is coupled to DA 1 receptor and mice deficient in Gng7 exhibit down-regulation of DA 2 receptor (D2R) (Sasaki et al., 2013). In mPFC, CSD led to de-regulation of 20 genes (14 ↓ and 6 ↑). Again, the top canonical pathways enriched by these genes were immuno-inflammation pathways: IL-12 signalling and production of macrophages, and production of nitric oxide and reactive oxygen species in macrophages. One of the up-regulated genes in mPFC of CSD mice was *Prkcd*: Prkcd has been identified as a major mediator of TNF-induced degeneration of DA neurons (Gordon et al., 2012). Several recent human studies have reported increased expression of immuno-inflammation genes in depressed relative to healthy-control probands. These include a genome-wide expression study of peripheral blood mononuclear cells that identified increased *TNF* expression in depressed patients, who also displayed relatively high hemodynamic responses to sad versus happy faces in HIPP, AMYG, and mPFC (Savitz et al., 2013). *A post mortem* brain tissue microarray study reported

increased expression of genes belonging to pro-inflammatory and anti-inflammatory cytokine pathways in mPFC of depressed relative to healthy-control subjects (Shelton et al., 2011). Therefore, both peripheral biomarkers and central gene expression indicate that the refined CSD impacted on immuno-inflammation processes. As noted above, psychosocial stress is a potent activator of peripheral inflammation (Bierhaus et al., 2003; Pace et al., 2006) and several mechanisms have been either demonstrated or proposed to account for peripheral-to-central activation of immuno-inflammation in glial cells leading to altered neuronal functioning, including: cytokine passage and active transport through the blood-brain barrier, activation of endothelial cells and macrophages lining the cerebral vasculature, and passage of tryptophan catabolites to the brain (Dantzer et al., 2008; Miller et al., 2009). Furthermore, the impact of immuno-inflammation on monoamine neurotransmission – both DA and 5-HT - has been proposed as a major aetio-pathophysiological pathway for depression (Felger and Miller, 2012; Haroon et al., 2012).

With respect to genes that were de-regulated by CSD and are candidates for direct causal involvement in the depression-relevant behavioural states identified in CSD mice, it is important that for AMYG a number of these genes express proteins that are either mediators or regulators of DA function; specifically, *Drd2*, *Adora2a*, *Gpr88*, *Darpp-32*, *Rgs9*, *Slc29a4* and, as discussed above, *Gng7* (as well as *Prkcd* in mPFC). Dopamine is released into the basolateral (BLA) and central (CeA) AMYG in the presence of emotional stimuli, and both regions express non-overlapping populations of D1 and D2 receptors, primarily on glutamate projection neurons in BLA and GABA neurons in CeA (Perez de la Mora et al., 2012). D2 receptor expression is high in CeA, as it is in the inter-connected structures of the bed nucleus of the stria terminalis and nucleus accumbens (central extended AMYG) and the dorsal striatum-external pallidum (Alheid and Heimer, 1988). Systemic injection of a D2R antagonist decreased two-way active avoidance in rat (Reis et al., 2004) and injection into CeA increased fear in rat (de la Mora et al., 2010); these findings provide supportive evidence that decreased *Drd2* contributed to the increased freezing and decreased active avoidance observed in CSD mice. D2R interacts antagonistically with the adenosine receptor A2AR by forming heteromers (Boison et al., 2012). Given that both *Drd2* and *Adora2a* expression was decreased in CSD mice it is difficult to predict the functional consequences. Nonetheless, it is noteworthy that striatum-specific A2AR knockout mice exhibited hypo-activity and deficient two-way active avoidance (Singer et al., 2013), as observed here in CSD mice. *Adora2a* and *Gpr88* both exhibit enriched expression in the central extended AMYG and dorsal striatum (Becker et al., 2008); accordingly, the decreased expression observed in CSD relative to CON mice might well have occurred primarily in the CeA, indicating that future studies should extend analysis of CSD effects on gene expression to these striatal regions. In striatum, GPR88 is expressed on GABA medium spiny neurons also expressing D2R or D1R; *Gpr88* knockout mice exhibited impaired active avoidance (Quintana et al., 2012), as observed here in CSD mice. *Darpp-32* encodes a signalling phosphoprotein that is expressed in D2R and D1R neurons and is a major integrator of signals from neurotransmitters and neuromodulators targeting these neurons; again, it has been mainly studied in striatum but decreased expression here and, as observed in CSD mice, AMYG, would be expected to have important consequences for DA neuron function and behaviour (Bateup et al., 2010). *Rgs9* encodes a protein, Rgs9-2, that modulates D2R function: *Rgs9* knockout mice exhibited increased locomotor activation in response to amphetamine, indicating that

Rgs9-2 normally reduces D2R signalling (Rahman et al., 2003); however, in such mice there was no change in D2R levels, whereas in CSD mice the decrease in *Rgs9* might constitute a response to decreased D2R levels. *Slc29a4* encodes a protein that is expressed presynaptically by monoamine neurons and catalyses monoamine reuptake, and is moderately expressed in AMYG of mouse (Dahlin et al., 2007). *Slc29a4* was up-regulated in the AMYG of CSD mice such that they would be expected to exhibit reduced DA and 5-HT levels in the synaptic cleft relative to CON mice. Combining the current transcriptional evidence for decreased AMYG D2R function in CSD mice with the growing evidence that striatal D2R enhances motivation (Trifilieff et al., 2013), then this valid CSD model can be utilized to increase understanding of D2R-motivation mechanisms, in terms of the motivation to both exhibit behaviour that leads to relief from punishment and that leads to wanting and liking reward. Also with regards to the increased fatigue demonstrated in CSD mice, theory and evidence supports de-regulated DA-striatal functioning as a major underlying neuropathology (Capuron et al., 2007; Demyttenaere et al., 2005). The relevance of this model is further indicated by the human evidence that a polymorphism in *D2R* associated with reduced D2R density, particularly in dorsal striatum, increased risk of depression (Zou et al., 2012).

In summary, this mouse model for psychosocial stress induced hyper-reactivity to aversive events exhibits levels of both aetiological and face validity that are unusually high, and accordingly it constitutes a major step forward in the research field of animal models of depression. The subsequent identification of physiological and cortico-limbic transcriptome-expression characteristics of the model has put focus on stress-immuno-inflammation-dopamine dysfunction as an aetio-pathophysiological pathway and as a target for novel mechanisms of anti-depressant action.

# 6. ACKNOWLEDGEMENTS

We are extremely grateful to Werner Rust, Dagmar Knebel, Susanne Acker and Karsten Quast for expert technical assistance. Tobias Hildebrandt, German Leparc and Bastian Hengerer are employees of Boehringer Ingelheim Pharma GmbH & Co. KG. This research was funded by the Swiss National Science Foundation grants 31003A-130499 and 31003A-141137.

# 7.SUPPLEMENTAL DATA OF CHAPTER 2

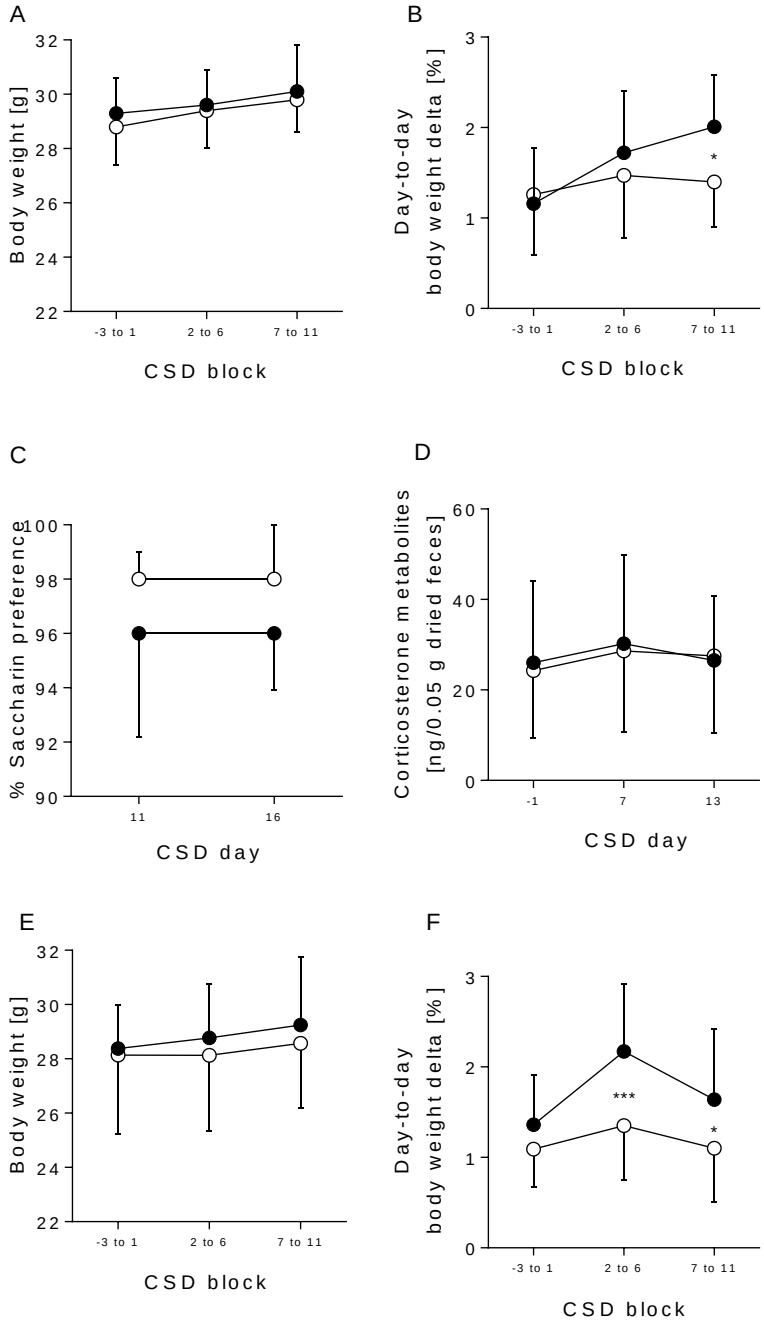



Figure S1: Effects of chronic social defeat on repeated physical measures. Expt. B (CON = 10 (open circles), CSD = 13 (filled circles)): (A) Absolute body weight. (B) Percent day-to-day body weight delta (abs(BW day n – BW day n-1))/(BW day n-1)*100. (C) Percent saccharin preference at [0.15 %] (day 11) and [0.5 %] (day 16). (D) Faecal corticosterone metabolite concentrations. Expt. C (CON = 12, CSD = 12): (E) Absolute body weight. (F) Percent day-to-day body weight delta. Values are means +/- SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0005$.

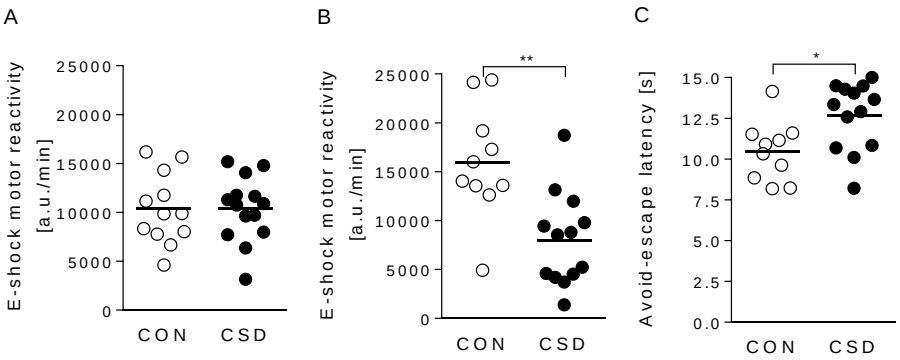



Figure S2: Short- and long-term effects of chronic social defeat in two-way active escape-avoidance test. Short-term (Expt A, CON = 13 (open circles), CSD = 14 (filled circles)): (A) Mean motor reactivity to escapable electroshock. Long-term (Expt B, CON = 10 (open circles), CSD = 13 (filled circles)): (B) Mean motor reactivity to escapable electroshock. (C) Mean latency to exhibit an avoid-escape response. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0005$.

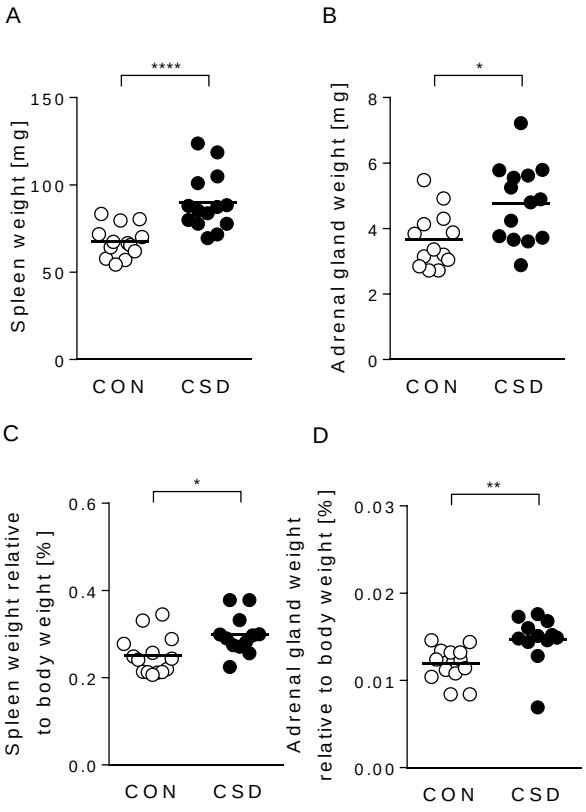



Figure S3: Short-term effects of chronic social defeat on physiological measures. Expt. A (CON = 13 (open circles), CSD = 14 (filled circles)): (A) Absolute spleen weight. (B) Absolute weight of adrenal glands. Expt. C (CON = 12 (open circles), CSD = 12 (filled circles)): (C) Spleen weight relative to body weight. (D) Adrenal gland weight relative to body weight. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0005$.

**Table S1. Cytokine-regulated genes exhibiting altered expression in CSD versus control mice in ventral hippocampus**

| Gene | | Up-/down-regulation | Fold change[1] | Upstream cytokine[2] |
|---|---|---|---|---|
| *Ace* | Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1 | ↑ | 1.6 | TNF |
| *Enpp2* | Ectonucleotide pyrophosphatase/phosphodiesterase 2 | ↑ | 1.9 | IL6, TNF |
| *Igf2* | Insulin-like growth factor 2 (somatomedin A) | ↑ | 1.6 | IL6, TNF |
| *Kl* | Klotho | ↑ | 1.7 | TNF |
| *Prox1* | Prospero homeobox 1 | ↑ | 1.4 | IL3 |
| *Tnfrsf25* | Tumor necrosis factor receptor superfamily, member 25 | ↑ | 1.7 | IL6 |
| *Ar* | Androgen receptor | ↓ | 1.4 | TNF |
| *Dcn* | Decorin | ↓ | 1.7 | IL6 |
| *Egr1* | Early growth response 1 | ↓ | 1.6 | IL6, TNF, IL3 |
| *Nov* | Nephroblastoma overexpressed | ↓ | 1.7 | TNF, IL3 |

[1] *q*-values for fold change = 0.005 in all cases

[2] upstream analysis conducted using Ingenuity Pathway Analysis

**Table S2. NMDA receptor-regulated genes exhibiting altered expression in CSD versus control mice in medial prefrontal cortex**

| Gene | | Up-/down-regulation | Fold change | q-value | Reference[1] |
|---|---|---|---|---|---|
| *Arc* | Activity-regulated cytoskeleton-associated protein | ↑ | 1.5 | 0.0126 | Link et al. (1995) |
| *Fos* | FBJ murine osteosarcoma viral oncogene homolog | ↑ | 1.4 | 0.0469 | Leveque et al. (2000) |
| *Penk* | Proenkephalin | ↑ | 1.7 | 0.0126 | Leveque et al. (2000) |

[1] upstream analysis conducted using Ingenuity Pathway Analysis

**Table S3. Genes exhibiting up-regulated expression in CSD versus control mice in ventral hippocampus (vHIPP), amygdala (AMYG) and medial prefrontal cortex (mPFC)**

| Brain region | | Gene ID | Gene | RPKM control | RPKM CSD | Fold change | *q*-value |
|---|---|---|---|---|---|---|---|
| | mPFC | ENSMUSG00000022602 | *Arc* | 14.961 | 22.587 | 1.5 | 0.0126 |
| AMYG/mPFC | AMY | ENSMUSG00000022602 | *Arc* | 17.472 | 24.853 | 1.4 | 0.0030 |
| | AMY | ENSMUSG00000050822 | *Slc29a4* | 4.429 | 6.282 | 1.4 | 0.0030 |
| AMYG/vHIPP | vHIPP | ENSMUSG00000050822 | *Slc29a4* | 9.567 | 14.122 | 1.5 | 0.0053 |
| AMYG | AMYG | ENSMUSG00000048218 | *Amigo2* | 4.099 | 7.621 | 1.9 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000025128 | *Bhlhe22* | 18.400 | 28.965 | 1.6 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000067578 | *Cbln4* | 6.494 | 9.973 | 1.5 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000019929 | *Dcn* | 3.786 | 8.339 | 2.2 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000047415 | *Gpr68* | 5.057 | 7.206 | 1.4 | 0.0155 |
| AMYG | AMYG | ENSMUSG00000052684 | *Jun* | 25.926 | 36.914 | 1.4 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000046523 | *Kctd4* | 18.093 | 28.197 | 1.6 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000026344 | *Lypd1* | 257.074 | 375.206 | 1.5 | 0.0120 |
| AMYG | AMYG | ENSMUSG00000067786 | *Nnat* | 254.146 | 364.756 | 1.4 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000030551 | *Nr2f2* | 32.036 | 45.592 | 1.4 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000050505 | *Pcdh20* | 8.131 | 11.709 | 1.4 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000092035 | *Peg10* | 15.679 | 21.241 | 1.5 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000068744 | *Psrc1* | 6.309 | 9.971 | 1.6 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000026395 | *Ptprc* | 4.378 | 6.616 | 1.5 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000029641 | *Rasl11a* | 5.674 | 8.425 | 1.5 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000030259 | *Rassf8* | 5.431 | 8.282 | 1.5 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000039087 | *Rreb1* | 7.629 | 11.544 | 1.5 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000028360 | *Slc44a5* | 4.950 | 7.657 | 1.5 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000050074 | *Spink8* | 10.131 | 16.526 | 1.6 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000039239 | *Tgfb2* | 5.700 | 8.521 | 1.5 | 0.0030 |
| vHIPP | vHIPP | ENSMUSG00000026051 | *1500015O10Rik* | 3.353 | 8.080 | 2.4 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000020681 | *Ace* | 4.309 | 6.845 | 1.6 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000036907 | *C1ql2* | 29.315 | 43.955 | 1.5 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000003657 | *Calb2* | 31.411 | 46.448 | 1.5 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000025370 | *Cdh9* | 9.296 | 14.726 | 1.6 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000030270 | *Cpne9* | 3.053 | 5.402 | 1.8 | 0.0183 |
| vHIPP | vHIPP | ENSMUSG00000022425 | *Enpp2* | 64.721 | 125.099 | 1.9 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000069919 | *Hba-a1* | 6.688 | 9.821 | 1.5 | 0.0213 |
| vHIPP | vHIPP | ENSMUSG00000069917 | *Hba-a2* | 4.284 | 6.935 | 1.6 | 0.0147 |
| vHIPP | vHIPP | ENSMUSG00000052305 | *Hbb-b1* | 19.204 | 28.382 | 1.5 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000048583 | *Igf2* | 18.528 | 30.015 | 1.6 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000058488 | *Kl* | 5.237 | 8.974 | 1.7 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000090291 | *Lrrc10b* | 18.318 | 25.828 | 1.4 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000040998 | *Npnt* | 5.872 | 8.757 | 1.5 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000031557 | *Plekha2* | 5.311 | 8.125 | 1.5 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000035486 | *Plk5* | 5.401 | 8.560 | 1.6 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000010175 | *Prox1* | 12.357 | 17.445 | 1.4 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000040929 | *Rfx3* | 11.507 | 16.908 | 1.5 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000023886 | *Smoc2* | 11.647 | 16.504 | 1.4 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000024793 | *Tnfrsf25* | 4.713 | 8.042 | 1.7 | 0.0053 |
| mPFC | mPFC | ENSMUSG00000021250 | *Fos* | 4.570 | 6.409 | 1.4 | 0.0469 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| mPFC | mPFC | ENSMUSG00000059991 | *Nptx2* | 12.092 | 19.531 | 1.6 | 0.0126 |
| mPFC | mPFC | ENSMUSG00000045573 | *Penk* | 21.351 | 36.508 | 1.7 | 0.0126 |
| mPFC | mPFC | ENSMUSG00000021948 | *Prkcd* | 3.521 | 5.862 | 1.7 | 0.0126 |
| mPFC | mPFC | ENSMUSG00000046480 | *Scn4b* | 4.453 | 6.706 | 1.5 | 0.0126 |

**Table S4. Genes exhibiting down-regulated expression in CSD versus control mice in ventral hippocampus (vHIPP), amygdala (AMYG) and medial prefrontal cortex (mPFC)**

| Brain region | | Gene ID | Gene | RPKM control | RPKM CSD | Fold change | *q*-value |
|---|---|---|---|---|---|---|---|
| AMYG mPFC vHIPP | mPFC | ENSMUSG00000088595 | *5_8S_rRNA* | 10.753 | 1.972 | 5.45 | 0.0126 |
| | AMYG | ENSMUSG00000088595 | *5_8S_rRNA* | 14.137 | 1.664 | 8.50 | 0.0030 |
| | vHIPP | ENSMUSG00000088595 | *5_8S_rRNA* | 10.558 | 1.668 | 6.33 | 0.0053 |
| AMYG mPFC vHIPP | mPFC | ENSMUSG00000065037 | *7SK* | 7.024 | 2.823 | 2.49 | 0.0126 |
| | AMYG | ENSMUSG00000065037 | *7SK* | 7.155 | 2.801 | 2.55 | 0.0030 |
| | vHIPP | ENSMUSG00000065037 | *7SK* | 6.474 | 2.745 | 2.36 | 0.0053 |
| AMYG mPFC vHIPP | mPFC | ENSMUSG00000045999 | *AY036118* | 260.950 | 87.270 | 2.99 | 0.0126 |
| | AMYG | ENSMUSG00000045999 | *AY036118* | 274.707 | 97.491 | 2.82 | 0.0030 |
| | vHIPP | ENSMUSG00000045999 | *AY036118* | 262.753 | 79.583 | 3.30 | 0.0053 |
| AMYG mPFC vHIPP | mPFC | ENSMUSG00000035202 | *Lars2* | 71.419 | 38.563 | 1.85 | 0.0126 |
| | AMYG | ENSMUSG00000035202 | *Lars2* | 75.903 | 45.526 | 1.67 | 0.0030 |
| | vHIPP | ENSMUSG00000035202 | *Lars2* | 78.720 | 47.142 | 1.67 | 0.0053 |
| AMYG mPFC vHIPP | mPFC | ENSMUSG00000092984 | *Mir5115* | 88.290 | 43.319 | 2.04 | 0.0126 |
| | AMYG | ENSMUSG00000092984 | *Mir5115* | 103.499 | 56.274 | 1.84 | 0.0030 |
| | vHIPP | ENSMUSG00000092984 | *Mir5115* | 107.950 | 52.222 | 2.07 | 0.0053 |
| AMYG mPFC | mPFC | ENSMUSG00000022548 | *Apod* | 83.409 | 56.379 | 1.48 | 0.0126 |
| | AMYG | ENSMUSG00000022548 | *Apod* | 60.026 | 42.585 | 1.41 | 0.0030 |
| AMYG mPFC | mPFC | ENSMUSG00000020774 | *Aspa* | 9.687 | 6.722 | 1.44 | 0.0225 |
| | AMYG | ENSMUSG00000020774 | *Aspa* | 7.626 | 5.421 | 1.41 | 0.0079 |
| AMYG mPFC | mPFC | ENSMUSG00000027375 | *Mal* | 90.783 | 60.801 | 1.49 | 0.0126 |
| | AMYG | ENSMUSG00000027375 | *Mal* | 65.611 | 45.313 | 1.45 | 0.0030 |
| AMYG mPFC | mPFC | ENSMUSG00000032517 | *Mobp* | 254.176 | 171.536 | 1.48 | 0.0309 |
| | AMYG | ENSMUSG00000032517 | *Mobp* | 182.943 | 127.100 | 1.44 | 0.0030 |
| AMYG mPFC | mPFC | ENSMUSG00000050121 | *Opalin* | 8.313 | 5.399 | 1.54 | 0.0126 |
| | AMYG | ENSMUSG00000050121 | *Opalin* | 5.836 | 3.631 | 1.61 | 0.0030 |
| AMYG mPFC | mPFC | ENSMUSG00000015090 | *Ptgds* | 159.973 | 113.552 | 1.41 | 0.0126 |
| | AMYG | ENSMUSG00000015090 | *Ptgds* | 95.532 | 62.656 | 1.52 | 0.0030 |
| AMYG vHIPP | AMYG | ENSMUSG00000065922 | *n-R5-8s1* | 5.936 | 1.380 | 4.30 | 0.0030 |
| | vHIPP | ENSMUSG00000065922 | *n-R5-8s1* | 5.167 | 0.905 | 5.71 | 0.0053 |
| AMYG | AMYG | ENSMUSG00000084890 | *A830036E02Rik* | 12.318 | 8.695 | 1.42 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000020178 | *Adora2a* | 39.322 | 21.111 | 1.86 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000078958 | *Atp6ap1l* | 5.336 | 2.657 | 2.01 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000000861 | *Bcl11a* | 19.171 | 13.491 | 1.42 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000023274 | *Cd4* | 5.102 | 2.418 | 2.11 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000046159 | *Chrm3* | 8.038 | 5.405 | 1.49 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000019997 | *Ctgf* | 25.467 | 16.188 | 1.57 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000061718 | *Darpp-32* | 237.205 | 141.374 | 1.68 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000032259 | *Drd2* | 18.902 | 10.534 | 1.79 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000009216 | *Fam163b* | 36.595 | 24.422 | 1.50 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000029054 | *Gabrd* | 18.366 | 11.142 | 1.65 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000048240 | *Gng7* | 91.722 | 63.788 | 1.44 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000046922 | *Gpr6* | 8.224 | 4.088 | 2.01 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000068696 | *Gpr88* | 71.788 | 39.949 | 1.80 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000046182 | *Gsg1l* | 17.654 | 10.260 | 1.72 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000051022 | *Hs3st1* | 9.216 | 6.360 | 1.45 | 0.0030 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| AMYG | AMYG | ENSMUSG00000034997 | *Htr2a* | 5.699 | 3.939 | 1.45 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000037679 | *Inf2* | 32.367 | 21.208 | 1.53 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000037624 | *Kcnk2* | 13.496 | 9.097 | 1.48 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000090291 | *Lrrc10b* | 19.161 | 11.162 | 1.72 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000026765 | *Lypd6b* | 7.754 | 4.989 | 1.55 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000040258 | *Nxph4* | 5.197 | 3.059 | 1.70 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000090223 | *Pcp4* | 325.298 | 217.974 | 1.49 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000038370 | *Pcp4l1* | 54.503 | 34.383 | 1.59 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000023868 | *Pde10a* | 38.252 | 26.137 | 1.54 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000017491 | *Rarb* | 7.408 | 5.193 | 1.43 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000032946 | *Rasgrp2* | 34.295 | 20.586 | 1.67 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000042453 | *Reln* | 14.411 | 10.147 | 1.42 | 0.0138 |
| AMYG | AMYG | ENSMUSG00000020599 | *Rgs9* | 28.862 | 14.761 | 1.96 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000046480 | *Scn4b* | 37.309 | 15.126 | 2.47 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000052133 | *Sema5b* | 9.332 | 6.288 | 1.48 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000044813 | *Shb* | 5.152 | 3.649 | 1.41 | 0.0220 |
| AMYG | AMYG | ENSMUSG00000030688 | *Stard10* | 38.296 | 26.358 | 1.45 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000061762 | *Tac1* | 43.398 | 22.810 | 1.90 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000071234 | *Tmem90a/SYNDIG1L* | 16.047 | 8.463 | 1.90 | 0.0030 |
| AMYG | AMYG | ENSMUSG00000062151 | *Unc13c* | 9.057 | 5.481 | 1.65 | 0.0030 |
| vHIPP | vHIPP | ENSMUSG00000097789 | *AC129605.1/Gm2115* | 28.558 | 19.843 | 1.44 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000078137 | *Ankrd63* | 7.013 | 3.596 | 1.95 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000046532 | *Ar* | 5.550 | 3.899 | 1.42 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000017978 | *Cadps2* | 39.851 | 28.209 | 1.41 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000060402 | *Chst8* | 11.340 | 7.636 | 1.49 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000052560 | *Cpne8* | 9.797 | 6.252 | 1.57 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000019929 | *Dcn* | 44.317 | 26.463 | 1.67 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000075707 | *Dio3* | 5.241 | 3.039 | 1.72 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000040856 | *Dlk1* | 5.549 | 3.210 | 1.73 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000038418 | *Egr1* | 21.222 | 13.301 | 1.60 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000004151 | *Etv1* | 16.751 | 11.922 | 1.41 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000026841 | *Fibcd1* | 57.023 | 30.129 | 1.89 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000033316 | *Galnt9* | 54.938 | 39.213 | 1.40 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000040836 | *Gpr161* | 15.670 | 10.917 | 1.44 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000039706 | *Ldb2* | 21.073 | 14.055 | 1.50 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000038793 | *Lefty1* | 5.169 | 2.846 | 1.82 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000028184 | *Lphn2* | 10.808 | 7.164 | 1.51 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000003746 | *Man1a* | 17.329 | 12.061 | 1.44 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000041708 | *Mpped1* | 103.821 | 72.814 | 1.43 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000005125 | *Ndrg1* | 48.680 | 33.154 | 1.47 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000027977 | *Ndst3* | 7.412 | 5.106 | 1.45 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000037362 | *Nov* | 85.742 | 49.584 | 1.73 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000075270 | *Pde11a* | 9.312 | 6.490 | 1.43 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000027674 | *Pex5l* | 90.665 | 64.210 | 1.41 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000090125 | *Pou3f1* | 18.616 | 11.094 | 1.68 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000028909 | *Ptpru* | 25.736 | 15.855 | 1.62 | 0.0053 |
| vHIPP | vHIPP | ENSMUSG00000050074 | *Spink8* | 34.428 | 22.560 | 1.53 | 0.0053 |
| mPFC | mPFC | ENSMUSG00000013523 | *Bcas1* | 49.974 | 35.271 | 1.42 | 0.0126 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| mPFC | mPFC | ENSMUSG00000043448 | *Gjc2* | 6.617 | 4.660 | 1.42 | 0.0126 |
| mPFC | mPFC | ENSMUSG00000093077 | *Mir5105* | 917.167 | 612.185 | 1.50 | 0.0126 |

# Chapter 3

**A genetic mouse model of reduced serotonin reuptake exhibits attenuated inflammatory and fear conditioning responses to adult psychosocial stress: integrating the serotonin and cytokine hypotheses of depression**

Damiano Azzinnari[1, 5], Hannes Sigrist[1], Giorgio Bergamini[1, 5], Boris Ferger[2], Klaus-Peter Lesch[3], Erich Seifritz[4, 5], Christopher R. Pryce[1, 5]

1 Preclinical Laboratory for Translational Research into Affective Disorders, Department of Psychiatry, Psychotherapy and Psychosomatics, Psychiatric Hospital, University of Zurich, Switzerland

2 CNS Diseases Research, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany

3 Molecular Psychiatry, Laboratory of Translational Neuroscience, Department of Psychiatry, Psychosomatics & Psychotherapy, University of Würzburg, Würzburg, Germany

4 Department of Psychiatry, Psychotherapy and Psychosomatics, Psychiatric Hospital, University of Zurich, Switzerland

5 Neuroscience Center Zurich, University of Zurich and ETH Zurich, Switzerland

# 1. SUMMARY

Interaction between the serotonin (5-HT) transporter (5-HTT) genotype and life stress events provides the most robust evidence to-date for gene-environment (G-E) interaction in the aetiology of depression. Nonetheless the evidence is controversial and the understanding of underlying mechanisms is scant, such that valid animal models are essential. Adult male wildtype (WT) and heterozygous (HET) littermates of a 5-HTT knockout (KO) mouse strain were each assigned to either psychosocial emotional stress as chronic social defeat (CSD) or control handling (CON), and studied in terms of monoamine neurochemistry, immuno-inflammation, endocrinology, and emotional behaviour. 5-HTT HET KO mice exhibited endophenotypes (HET-CON vs. WT-CON) of increased tissue levels of 5-HT and dopamine (DA) and their respective metabolites in nucleus accumbens, and increased basal plasma corticosterone. 5-HTT HET KO mice exhibited increased resilience to CSD: In WT mice, CSD induced increased basal plasma TNF levels and splenomegaly, and increased the acquisition of fear conditioned freezing to a discrete tone predicting electroshock. In contrast, none of these depression-relevant state markers were exhibited by HET-CSD relative to HET-CON mice. That endophenotypes of increased central 5-HT and DA activity are associated with absence of stress-induced emotional hyper-reactivity is consistent with the monoamine hypothesis of depression. That increased basal corticosterone and absence of stress-induced immuno-inflammation are associated with absence of emotional hyper-reactivity is consistent with the inflammation hypothesis of depression. Furthermore, these different HET-dependent pathways could be inter-dependent. The present G-E mouse model provides both translational support for the growing human evidence that decreased 5-HTT function increases stress resilience in adulthood, and a system with which to identify the responsible mechanisms and therefore targets for novel anti-depressant treatments.

# 2. INTRODUCTION

Two major hypotheses of depression aetio-pathophysiology are the serotonin (5-HT) deficiency hypothesis (Ruhe et al., 2007; Sharp and Cowen, 2011) and the pro-inflammatory cytokine hypothesis (Dantzer et al., 2008; Maes et al., 2009; Miller et al., 2009). The 5-HT deficiency hypothesis is based largely on the serendipitous discovery of antidepressant drugs that act on 5-HT neurotransmission, including current-generation selective 5-HT reuptake inhibitors (SSRIs), as well as physiological biomarkers including low blood levels of the 5-HT precursor tryptophan and low cerebrospinal fluid levels of 5-HT metabolites (Saveanu and Nemeroff, 2012; Sharp and Cowen, 2011). The cytokine hypothesis is based on association of depression with increased plasma levels of pro-inflammatory cytokines including tumor necrosis factor (TNF) and interleukin-6 (IL-6), high co-morbidity with autoimmune disorders, depression remission when autoimmune patients and depressed patients are treated with anti-inflammatory medication, high depression risk in patients of disorders requiring cytokine treatment, cytokine-gene polymorphisms as risk factors for depression, and up-regulation of cytokine genes in the brain in depression (Dantzer et al., 2008; Dantzer et al., 2011; Maes et al., 2009; Miller et al., 2009; Raison et al., 2013). Further to these two hypotheses of depression aetio-pathophysiology, there are several important lines of evidence inter-linking them, including increased pro-inflammatory cytokine levels leading to reduced 5-HT synthesis (Dantzer, 2009; Maes et al., 2011), and 5-HT suppression of pro-inflammatory cytokines and 5-HT stimulation of splenic T cell proliferation and anti-inflammatory cytokine synthesis (Kubera et al., 2000; Kubera et al., 2005). Intriguingly, it has also recently been proposed that increased cytokine release is essential to SSRI mechanism of action/efficacy, in direct contradiction to the cytokine hypothesis (Hernandez et al., 2013; Warner-Schmidt et al., 2011).

There exists a major paradox in the link between 5-HT and depression: whereas blocking the 5-HT transporter (5-HTT) is the basis of SSRI antidepressant action (Cowen, 2008), a 5-HTT gene polymorphism – namely the “short” (S) deletion versus “long” (L) insertion at the 5-HTT-linked polymorphic region (5-HTTLPR) - that, similar to SSRIs, reduces 5-HT reuptake (Murphy and Lesch, 2008), is currently the best-described genetic risk factor for depression (Lesch and Mossner, 1998). The S allele of the 5-HTT gene is associated with personality endophenotypes including anxiety and neuroticism (Lesch et al., 1996). Furthermore, it is a risk factor in interaction with environmental stress (GxE) (Caspi et al., 2010; Caspi et al., 2003). Meta-analysis yields strong statistical support for association between S x early-life stress (ELS) and depression, suggesting a neurodevelopmental process. However, there is marginal support for association between S x adult stress (AS) and depression (Karg et al., 2011). Given the high likelihood that the populations included in the studies of AS association with depression would also have experienced a representative level of ELS, the dual findings of S x ELS high association and S x AS low association is actually consistent with net S x AS negative association. That is, the S allele and a phenotype of low 5-HT reuptake are protective against AS leading to depression. This integrated interpretation of the two meta-analysis findings and the resultant hypothesis has, to our knowledge, not been proposed previously but is certainly worthy of investigation. This is not least because an S x AS interaction associated with resilience to depression

would resolve the paradox of low 5-HTT function = depression risk factor versus SSRI = mechanism of antidepressant action. Indeed, there already exists some supportive evidence, including: (1) The increase in new onset of depression following stress in teenager years tends to be greater in LL than SS carriers, and this despite the increase in chronic depression following early-life stress being greater in SS than LL carriers (Brown et al., 2013). (2) Individuals, including those exposed to pro-inflammatory cytokine medication, who have high 5-HT activity exhibit lower pro-inflammatory interleukin-6 levels and lower depression scores (Raison et al., 2009). (3) Globally, in geographical regions with a relatively high historical prevalence of pathogens the frequency of the S allele in the current population is also relatively high and the prevalence of depression is relatively low, suggesting that high 5-HT activity is protective against infection and depression (Chiao and Blizinsky, 2010).

To provide a genetic mouse model of some relevance to the human S 5-HTTLPR polymorphism, a 5-HTT null mutant mouse strain on a C57BL/6J background was generated (Bengel et al., 1998). Compared to wildtype (WT) mice, heterozygous 5-HTT mutant (HET) mice have decreased 5-HTT expression and increased 5-HT levels in various brain regions (Bengel et al., 1998; Mathews et al., 2004). A number of behavioural endophenotypes of relevance to depression risk and resilience have been reported for HET relative to WT mice, including: increased anxiety in approach-avoidance conflict tests (Jansen et al., 2010)and increased acquisition of learned helplessness in a two-way escape test (Pryce et al., 2012); but reduced sensitivity to omission of reward in an operant probabilistic reversal learning test (Ineichen et al., 2012). There was no evidence for a genotype effect on either acquisition or expression of fear conditioned freezing (Narayanan et al., 2011).

Psychosocial stressors are aetiological factors in human depression (Kendler et al., 1999), and animal models of depression that incorporate psychosocial emotional stress are likely to possess relatively high aetiological validity ((Krishnan and Nestler, 2008) Chapter 2). Mouse chronic social defeat (CSD) consists of exposure of adult C57BL/6 mice to daily brief physical attacks by different aggressive and dominant CD-1 mice and continuous sensory exposure to these aversive conspecifics. Depression-relevant behavioural effects of this psychosocial emotional stress include motor retardation in an open field test and a forced swim test (Kudryavtseva et al., 1991), increased anxiety in an elevated plus maze test (Avgustinovich et al., 2005; Krishnan et al., 2007), decreased reward sensitivity in a sucrose preference test (Yu et al., 2011), increased social avoidance (Krishnan et al., 2007), increased fear conditioned freezing to context, generalised helplessness, and increased fatigue (Chapter 2). At the physiological level, CSD induces immuno-inflammation in terms of increased plasma levels of pro-inflammatory cytokines and splenomegaly (Chapter 2). Centrally, CSD induces de-regulation of expression of genes in inflammatory pathways in the medial prefrontal cortex and amygdala, and in the latter it induces gene re-regulation consistent with compromised dopamine receptor 2 signalling (Chapter 2).

Based on the above evidence, the aim of the present study was to utilise a 5-HTT knockout mouse strain to investigate the effects of a genotype of reduced 5-HTT expression on trait/endophenotype status and on sensitivity/resilience to CSD in terms of the following measures: aversive emotional processing using classical fear conditioning; stress and anti-inflammatory endocrinology using plasma corticosterone; immuno-inflammation using plasma pro-inflammatory cytokines and spleen size; and tonic levels and turnover of serotonin and dopamine in the nucleus

accumbens. It was hypothesized that heterozygous knockout of 5-HTT would lead to increased resilience of adult mice to CSD-induced activation of immune-inflammation and, related to this, increased resilience to CSD-induced increased classical conditioning to an aversive stimulus.

# 3. EXPERIMENTAL PROCEDURES

## 3.1 Animals and maintenance

Breeding pairs of wildtype (WT) dams and heterozygous (HET) sires yielded WT and HET offspring. Male offspring were weaned at age 3 weeks and caged with littermates. At age 4 weeks an ear punch was taken, mice were genotyped (Bengel et al., 1998) and littermates of the same genotype were maintained together in groups of 2-3. The study was conducted with a total of 50 mice born to 12 breeding pairs. Mice were aged 9-12 weeks at study onset and weighed 23-30 g. The male CD-1 mice (Janvier, France, www.janvier-labs.com) used as aggressors in chronic social defeat (CSD) were aged 8 months, ex-breeders, and caged singly at study onset. Mice were maintained on a reversed 12:12 h light-dark cycle (lights off 07:00-19:00 h) in an individually-ventilated caging system (IVC) at 20-22 °C and 50-60% humidity. Cages were type 2L and contained woodchips, a sleep igloo and tissue bedding. Complete-pellet diet (Provimi, Kliba Ltd, Kaiseraugst, Switzerland) and water were available continuously and *ad libitum*. The study was conducted under a permit (110/2009) for animal experimentation issued by the Veterinary Office, Zurich, Switzerland, in accordance with the Animal Protection Act (1978) Switzerland. All efforts were made to minimize the number of mice used and any suffering of those mice that were used, including refinement of the published protocol for the stressor, CSD (Golden et al., 2011).

## 3.2 Chronic social defeat

Chronic social defeat comprised a CSD mouse being placed in the cage of an aggressive CD-1 mouse on CSD day 1 so that bouts of attack occurred. The mice remained together for a maximum of 60 sec of attack or for a maximum of 10 min. Thereafter the mice were separated by a central Plexiglas divider that was transparent and perforated to allow sensory communication but prevent physical attacks. After 24 h, the CSD mice were rotated between CD-1 mice cages and therefore confronted each day with a novel CD-1 aggressor. This procedure was repeated across 15 consecutive days between 14:00-17:00 h. Bite wounds were prevented by trimming the incisor teeth of the CD-1 mice every third day using rodent tooth-cutting forceps. The detailed CSD protocol is given in Chapter 2. Control mice remained in same-genotype littermate pairs, and were handled daily.

## 3.3 Experimental design

Wildtype and HET mice were each allocated at random to either the Control (CON; WT-CON N = 13, HET-CON N = 13) or the CSD condition (WT-CSD N = 14, HET-CSD N = 10). As schematised in Figure 1, CSD and CON-handling were conducted on days 1-15. At days 14, 15 and 17, respectively, mice were tested in terms of motor activity, discrete stimulus fear conditioning and hot plate pain sensitivity. Body weights (BW) were recorded daily. At day 18, mice were killed and trunk blood was collected for determination of plasma concentrations of pro-inflammatory cytokines and corticosterone, and spleen and adrenal glands were collected for weighing. Brains were fresh-frozen for region-specific determination of monoamine concentrations.

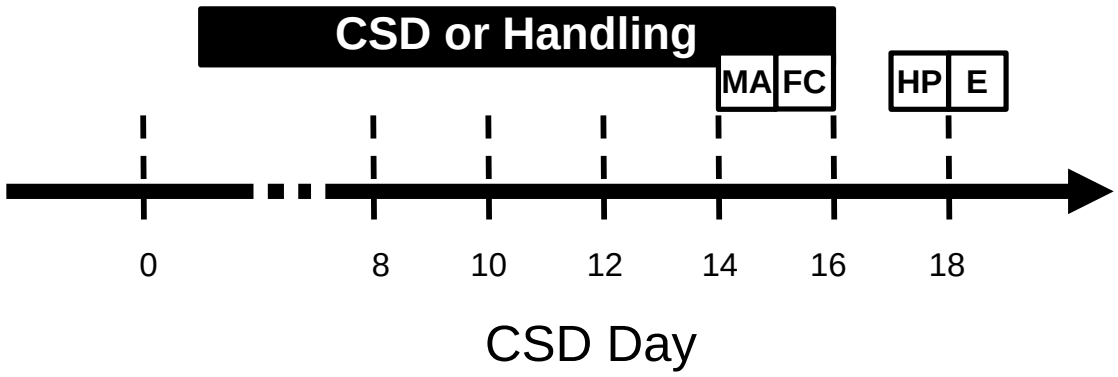



Figure 1. Experimental design. The effects of chronic social defeat (CSD) in wildtype and heterozygous mice of a serotonin transporter knockout strain were investigated in tests of motor activity (MA), discrete stimulus fear conditioning (FC) and hot plate (HP) pain sensitivity, followed by euthanasia (E) and *ex vivo* analyses.

## 3.4 Motor activity and CS fear conditioning

Behavioural testing was conducted under dim lighting in a room adjacent to the mouse holding room, between 08:30-12:00 h. Motor activity and discrete stimulus fear conditioning were conducted using a single multi-purpose system co-developed with the manufacturer (Multi Conditioning System, TSE Systems GmbH, Bad-Homburg, Germany) (details of which are given in (Pryce et al., 2012)).

**Motor activity.**

On day 14, the mouse was placed on the grid floor in the empty arena for 15 min without presentation of any further stimuli. Distance moved in the horizontal and vertical planes was recorded continuously. When the mouse did not make any movement for a period of at least 2 sec then this was recorded automatically as freezing. Distance moved/min (arbitrary units/min) and % time spent freezing were calculated.

**CS fear conditioning**

On day 15, the mouse was placed in the same arena and challenged with 10 trials of 12-sec tone (5 kHz, 85 dB, conditioned stimulus, CS) followed by 3 sec x 0.15 mA inescapable electroshock delivered at a constant inter-trial interval (ITI) of 50 sec. Distance moved during electroshock and % time spent freezing during CS were calculated.

## 3.5 Hot plate test

To assess pain sensitivity, the hot plate test was conducted using a programmable thermoelectric heating plate (Teca, Chicago IL, USA) set at 50 °C, with a transparent Plexiglas chamber placed onto the plate (Pryce et al., 2012). . On day 17, at 16:00-17:00 h, the mouse was placed inside the chamber and the latency (in sec) from the onset of the test until the first occurrence of one of the following behaviours was scored: licking a forepaw or hind paw, lifting a hind paw, jumping. The maximum test duration was 60 sec.

## 3.6 Blood, brain, spleen and adrenal gland collection

At day 18, for blood factor and fresh-fixed brain studies, mice were decapitated and trunk blood was collected in EDTA-coated tubes (Microvette 500 K3E, Sarstedt, Germany) and placed on ice. The brain was removed, snap-frozen on dry ice and stored at -80° C. Bloods were centrifuged at 3000 rpm and 4° C for 10 min, plasma aliquots transferred to cryotubes (Protein LoBind, Eppendorf, Germany) and stored at -80° C. Adrenal glands and spleen were removed, cleaned of fat and connective tissue and weighed.

## 3.7 Plasma cytokine measurement

Plasma titres of the pro-inflammatory cytokines IL-6 and TNF were measured using a multiplexed particle-based flow cytometric cytokine assay (described in (Marques-Vidal et al., 2011; Vignali, 2000)). Lower limits of detection for IL-6 and TNF were 0.5 pg/ml.

## 3.8 Corticosterone measurement

Plasma corticosterone titres were measured using an EIA kit (AssayMax Corticosterone ELISA kit; AssayPro, Saint Charles MO, USA). Plasma was diluted 1:50 in ELISA diluent and heated at 90° C for 10 min for transcortin denaturation. All further steps were performed according to the manufacturer's protocol.

## 3.9 Brain tissue monoamine determination

Frozen brains were sectioned coronally at 1.0 mm intervals using a stainless-steel brain matrix (Plastics One, model MMCS-1, Roanoke VA, USA) and single-edge blades (Apollo Herkenrath, model 10-100-063, Solingen, Germany). The nucleus accumbens (NAcc) was micro-dissected bilaterally from the corresponding section using a brain punch (∅ = 1.00 mm, Stoelting Europe, model 57397, Dublin, Ireland) and a mouse brain atlas (Franklin and Paxinos, 2008): 1 biopsy/hemisphere at bregma 1.5 to 0.5 ± 0.2 mm. Tissue mass was 1.5 ± 0.5 mg per sample. All micro-dissection steps were conducted at -18.0 °C. Brain biopsies were stored in 1.5 ml cryotubes (Protein LoBind) at -80.0 °C until tissue processing.

For tissue homogenization, ice-cooled perchloric acid (0.4 M, 350 µl) was added to each sample and ultrasonication was conducted for 10 sec (VibraCell, VCX130PB, Sonics and Materials, Inc., Newtown CT, USA) followed by centrifugation at 20,000×g for 10 min at 4°C (refrigerated microcentrifuge, CT15RE, VWR/Hitamachi, Lutterworth, UK). The supernatant was passed through a 0.22 µm filter (Minisart RC4, Sartorius AG, Göttingen, Germany) and kept on ice until analysis.

High performamce liquid chromatography (HPLC) and electrochemical detection (ED) were conducted for monoamine determination in brain tissue homogenates according to a published protocol (Oeckl and Ferger, 2012). Samples were analysed for serotonin (5-HT) and its metabolites 5-hydroxyindoleacetic acid (5-HIAA) and 3-methoxytyramine (3-MT), and dopamine (DA) and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). Isocratic separation of 5-HT and DA was carried out with a reversed-phase C18 column (YMC-Pack ODS-AQ, 100×2.1 mm, S-3 µm, YMC Europe GmbH, Dinslaken, Germany). An electrochemical cell with a glassy carbon electrode and an ISAAC Ag/AgCl reference electrode (VT-03, Antec, Zoeterwoude, Netherlands) was

used for ED. The mobile phase consisted of 1.7 mM 1-octanesulfonic acid sodium salt, 1.0 mM $Na_2EDTA \times 2\ H_2O$, 8.0 mM NaCl, 100 mM $NaH_2PO_4 \times 2\ H_2O$ (pH 3.80), mixed with 9.3% acetonitrile, and was delivered at a flow rate of 0.4 ml/min. Aliquots (20 µl) were injected onto the HPLC system by an autosampler with a cooling module set at 4°C; the loading order of samples was counterbalanced between groups. Concentrations of each monoamine/metabolite were calculated using an external standard calibration and expressed as ng/mg brain tissue. Monoamine turnover was calculated as: 5-HT = ([5-HIAA]+[3-MT])/[5-HT], DA = ([DOPAC]+[HVA])/[DA].

## 3.10 Statistical analysis

Statistical analysis was conducted using SPSS (version 20, SPSS Inc., Chicago IL, USA). Firstly, one way ANOVA with a between-subject factor of group (WT-CSD, WT-CON, HET-CSD, HET-CON) was conducted, and *a priori* pair-wise comparisons of interest were WT-CON vs HET-CON, WT-CSD vs WT-CON and HET-CSD vs HET-CON. In the case of a significant effect of group attributable to at least one of these pair-wise comparisons, a 2 x 2 factorial ANOVA with between-subject factors of 5-HTT genotype (WT, HET) x environment (CSD, CON) (GxE) was conducted. For body weight (BW) and % day-to-day BW delta (ΔBW), ANOVA was conducted with the between-subject factor of group and a within-subject factor of CSD day block (CSD day -3 to 1, 2 to 6, 7 to 11). *Post hoc* testing was conducted using the least significance difference (LSD) correction for multiple comparisons. Statistical significance was set at $p < 0.05$. Where an estimate of variance is given this is standard deviation (SD).

# 4. RESULTS

## 4.1 Repeated measures of physical status

The mean duration of attacks per CSD mouse was 47.0 ± 2.8 sec. The development of CSD effects was monitored across 5-day blocks of CSD day -3 to 1, 2 to 6 and 7 to 11. For absolute BW, there was a main effect of group ($F(3, 58) = 4.14$, $p < 0.01$; Fig. 2A). *Post hoc* analysis indicated that HET-CON mice were heavier than WT-CON mice ($p < 0.001$), and that this was the case for each time block ($p < 0.02$–$0.04$). For ΔBW, there was a main effect of group ($F(3, 59) = 3.67$, $p < 0.02$; Fig. 2B). *Post hoc* analysis indicated that ΔBW was increased by CSD in WT mice ($p < 0.005$); block-specific analysis indicated that ΔBW was greater in WT-CSD than WT-CON mice at days 2 to 6 ($p < 0.03$) and 7 to 11 ($p < 0.03$). There was no effect of CSD on ΔBW in HET mice ($p = 0.13$), and this also applied to each specific block ($p \geq 0.07$).

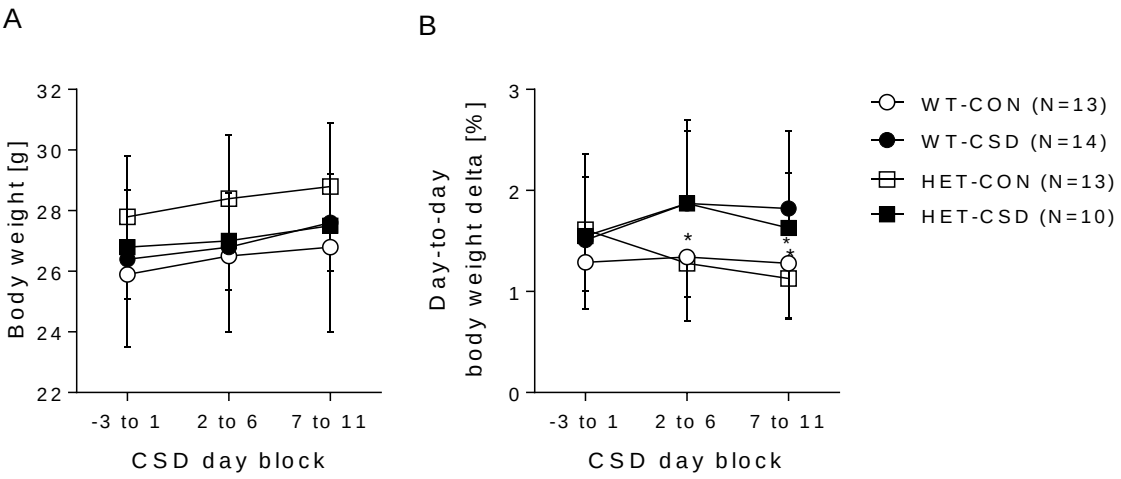



Figure 2. Effects of genotype and chronic social defeat on body weight measures. (A) Absolute body weight, (B) Percent day-to-day body weight delta (abs(BW day n – BW day n-1))/(BW day n-1)*100). Body weights were measured at 14:00 h each day and mean per mouse way calculated per time block. Values are mean +/- SD. * $p < 0.05$ for WT-CSD vs. WT-CON specifically.

## 4.2 Motor activity, CS fear conditioning and pain sensitivity

The effects of CSD were tested in terms of motor activity at day 14, CS fear conditioning and reactivity to electroshock at day 15, and pain sensitivity in the hot plate test at day 17. In the motor activity test, there was a main effect of group on distance moved/min ($F(3, 46) = 5.95$, $p < 0.002$): *post hoc* analysis indicated that WT-CON mice (6093±2272 arbitrary units (a.u.)/min) exhibited increased distance moved relative to HET-CON mice (4629±1050 a.u./min, $p < 0.02$) and WT-CSD mice (3961±1489 a.u./min, $p < 0.001$). In the GxE ANOVA there was no interaction effect ($p = 0.18$). In the same motor activity test, all groups spent a similar and low % time freezing ($p = 0.48$; Fig 3A). In the test of CS fear conditioning, there was a main effect of group on average % time freezing ($F(3, 44) = 6.92$, $p < 0.001$; Fig 3A). *Post hoc* analysis indicated that freezing was increased by CSD in WT mice ($p < 0.0005$) and not in HET mice ($p = 0.07$). In the GxE ANOVA there was not a significant interaction effect ($p = 0.17$).

In terms of motor reactivity to electroshock, there was no main effect of group ($p$ = 0.15; Fig. 3B). In the hot plate test there was a main effect of group ($F$(3, 41) = 2.86, $p$ < 0.05; Fig 3C): *post hoc* analysis indicated that HET-CSD mice exhibited decreased response latency relative to HET-CON mice ($p$ < 0.04). There was no significant interaction effect in the GxE ANOVA ($p$ = 0.47).

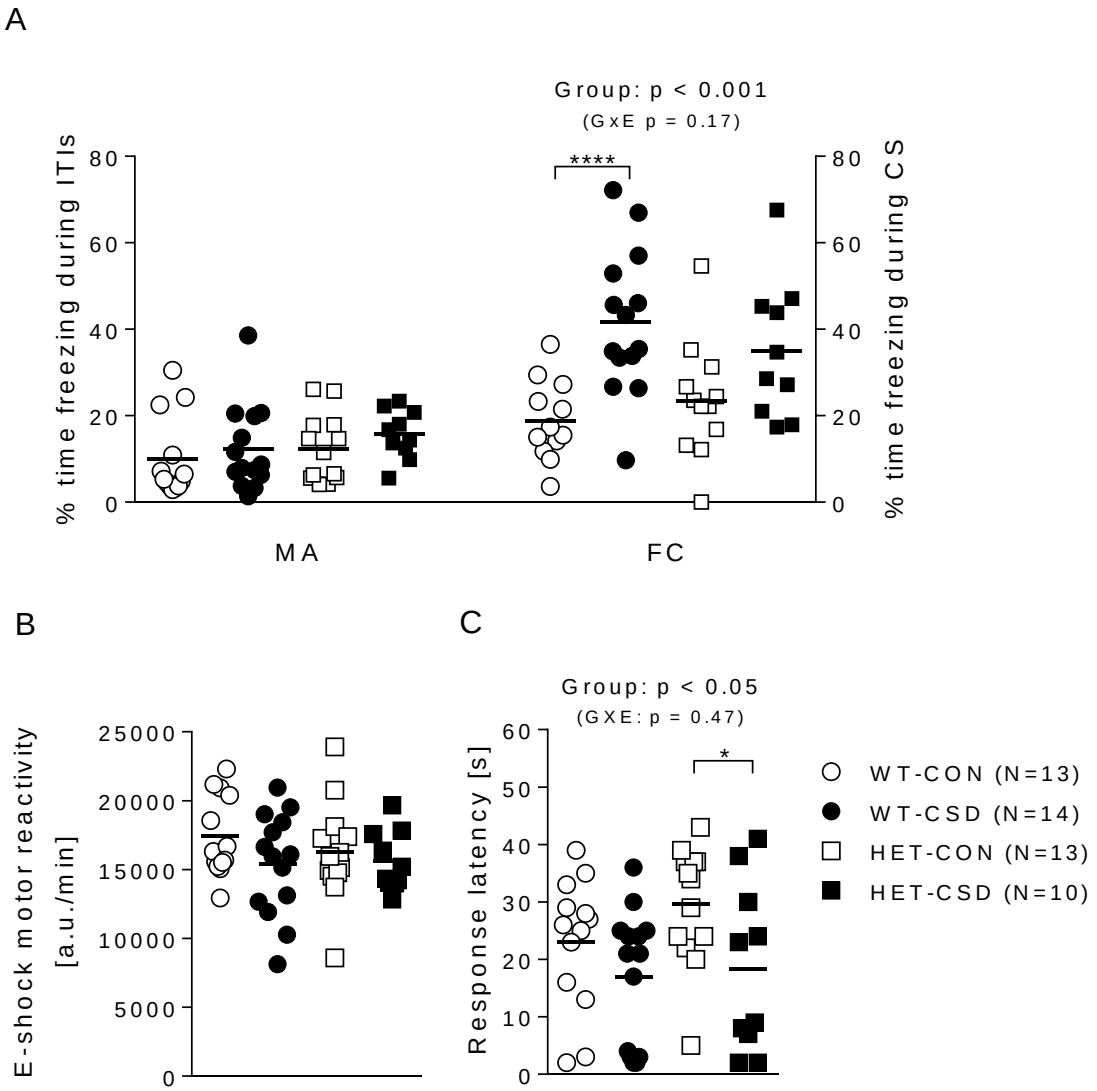



Figure 3. Effects of chronic social defeat on baseline and conditioned freezing. (A) Average per cent time freezing in the motor activity test (MA, day 14) and during the tone conditioned stimulus (CS) in the fear conditioning test (FC, day 15). (B) Average motor reactivity to electroshock in FC. (C) Pain response latency in the hot plate test (day 17). * $p$ < 0.05, **** $p$ < 0.0005.

## 4.3 Physiological status

Mice were decapitated at day 18 and trunk blood and tissues were collected. For spleen weight relative to BW there was a main effect of group ($F$(3, 46) = 3.44, $p$ < 0.02; Fig. 4A): in WT mice specifically, CSD induced an increase in relative spleen weight ($p$ < 0.004). There was a borderline non-significant interaction effect in the GxE ANOVA ($p$ = 0.08). For basal plasma concentrations of TNF, there was a main effect of group ($F$(3, 45) = 8.21, $p$ < 0.0005; Fig 4B): in WT mice specifically, CSD induced an increase in plasma TNF levels (p < 0.0005). There was a borderline non-significant

interaction effect in the GxE ANOVA ($p$ = 0.09). For basal plasma concentrations of IL-6, there was no main effect of group ($p$ = 0.37; Fig 4C). For adrenal gland weight relative to BW there was a main effect of group ($F$(3, 46) = 5.69, $p$ < 0.002; Fig 4D): CSD induced adrenomegaly in both WT mice ($p$ < 0.006) and HET mice ($p$ < 0.005). There was no interaction effect in the GxE ANOVA ($p$ = 0.79). For basal plasma concentrations of corticosterone, there was a main effect of group ($F$(3, 45) = 8.08, $p$ < 0.0005; Fig 4E): basal plasma corticosterone was increased in HET-CON relative to WT-CON mice ($p$ < 0.0005); CSD induced a decrease in plasma corticosterone in HET mice specifically ($p$ < 0.03). There was no GxE interaction effect ($p$ = 0.18).

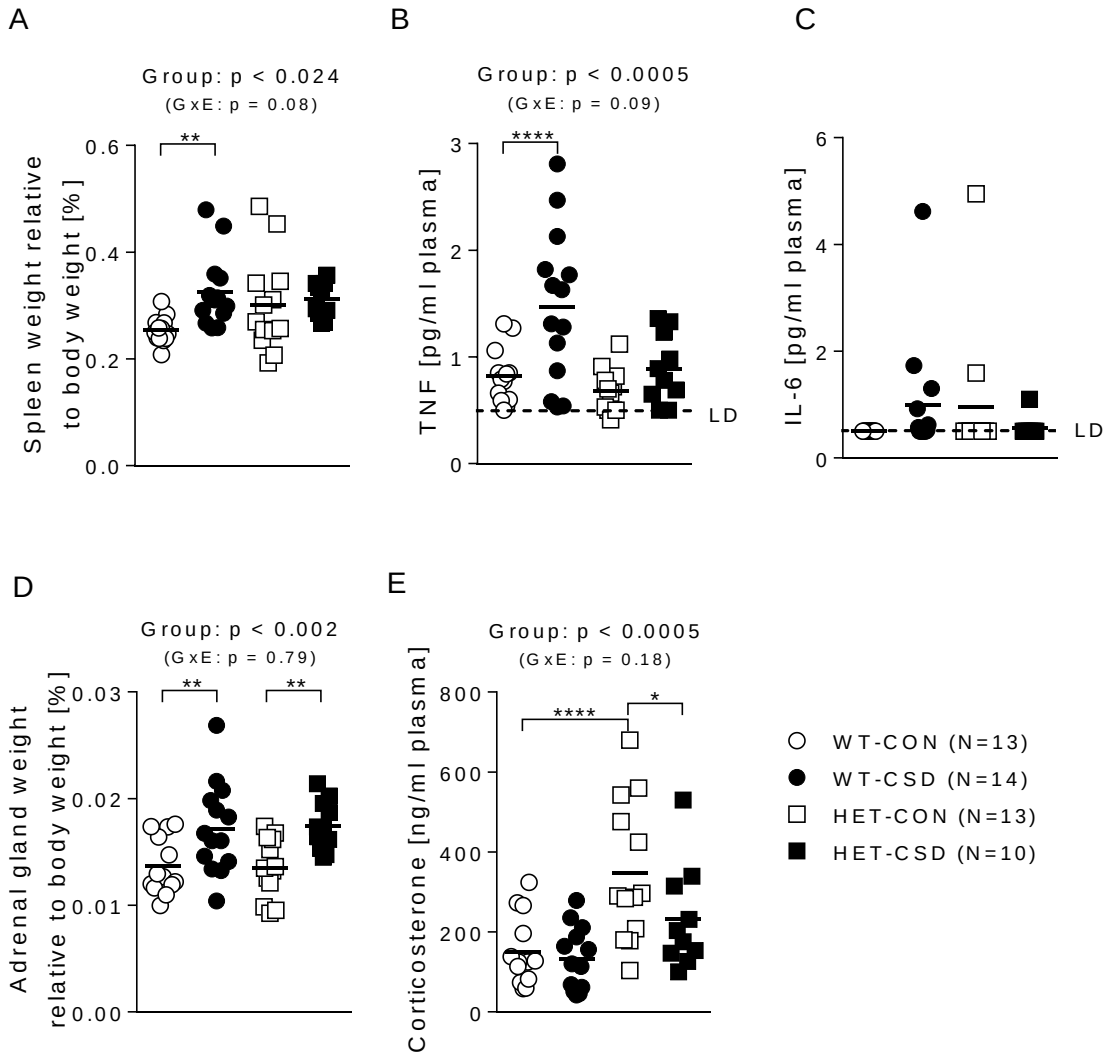



Figure 4. Effects of chronic social defeat on physiological measures. (A) Spleen weight relative to body weight. (B) Basal plasma concentration of tumor necrosis factor (TNF). (C) Basal plasma concentration of interleukin-6 (IL-6). (D) Total adrenal gland weight relative to body weight. (E) Basal plasma concentration of corticosterone. * $p$ < 0.05, ** $p$ < 0.01, **** $p$ < 0.0005. LD, limit of detection of immunoassay.

## 4.4 Monoamine levels in nucleus accumbens

From brains collected at day 18, the NAcc was dissected out and analysed in terms of its content of 5-HT, DA and their respective metabolites. For 5-HT ($F$(3, 37) = 5.79, $p$ < 0.002) as well as 5-HIAA $F$(3,

37) = 3.84, $p < 0.02$) and 3-MT ($F(3, 37) = 4.76$, $p < 0.007$), there was a main effect of group (Fig 5A-B). *Post hoc* analysis indicated in the case of each parameter that HET-CON mice exhibited increased concentrations relative to WT-CON mice ($p < 0.05$). There was no effect of CSD in either WT or HET mice; as would be predicted therefore, levels of 5-HT and its metabolites were also increased in HET-CSD relative to WT-CSD mice: 5-HT ($p < 0.001$), 5-HIAA ($p < 0.02$), 3-MT ($p < 0.004$). There was no main effect of group on 5-HT turnover ($p = 0.50$; Fig. 5C). For DA ($F(3, 37) = 3.89$, $p < 0.02$), DOPAC ($F(3, 36) = 7.88$, $p < 0.0005$) and HVA ($F(3, 36) = 5.28$, $p < 0.004$), there was a main effect of group (Fig 5D-E). *Post hoc* analysis indicated in the case of each parameter that HET-CON mice exhibited increased concentrations relative to WT-CON mice ($p < 0.004$-$0.03$). There was no effect of CSD in either WT or HET mice; as would be predicted therefore, levels of DA and its metabolites were also increased in HET-CSD relative to WT-CSD mice: DA ($p < 0.03$), 5-DOPCA ($p < 0.001$), HVA ($p < 0.005$). There was no main effect of group on DA turnover ($p = 0.45$; Fig 5F).

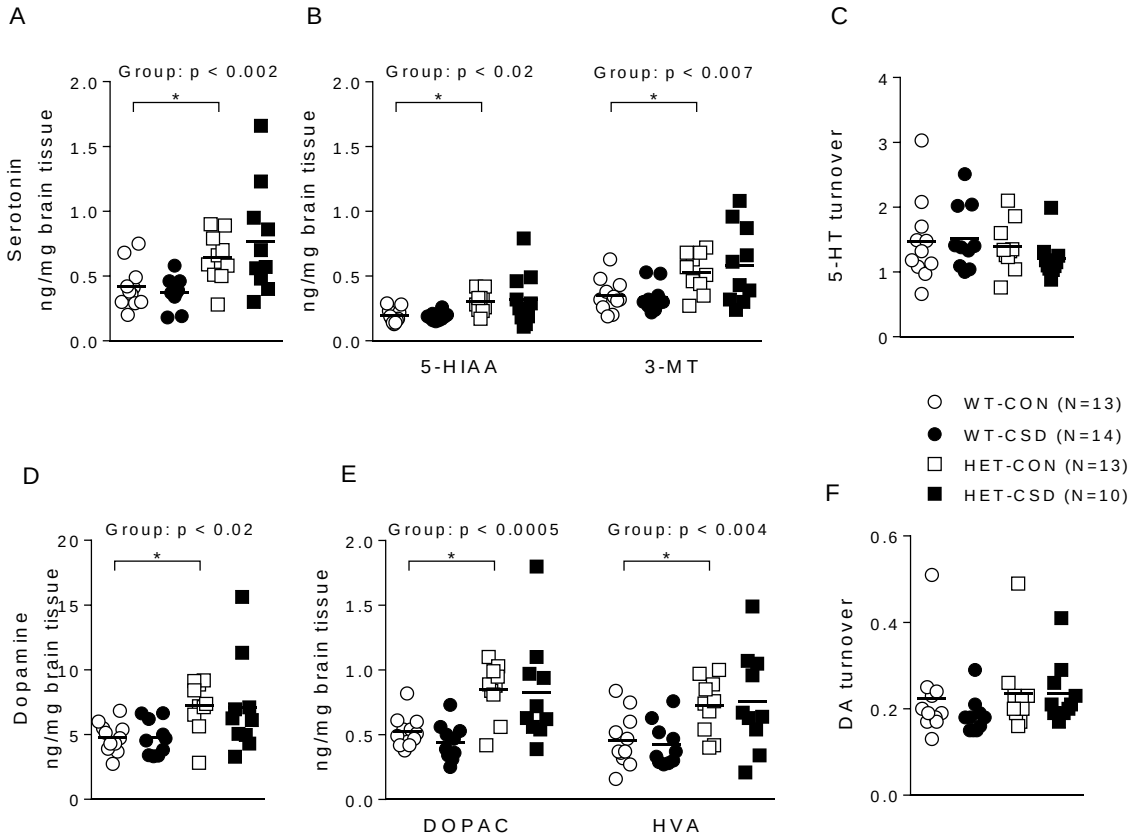



Figure 5. Effects of chronic social defeat on tissue concentrations of 5-HT and DA and respective metabolites in nucleus accumbens. (A) Serotonin, (B) Metabolites 5-hydroxyindoleacetic acid (5-HIAA) and 3-methoxytyramine (3-MT), (C) 5-HT turnover = ([5-HIAA] + [3-MT])/[5-HT]. (D) Dopamine, (E) Metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA), (F) DA turnover = ([DOPAC] + [HVA])/[DA]. * $p < 0.05$.

# 5. DISCUSSION

In humans, polymorphism in the 5-HTT gene leading to decreased 5-HTT expression and function is associated with endophenotypes of increased anxiety and neuroticism in adulthood and an increased long-term risk that early-life stress will lead to depression. However, the net GxE evidence is also consistent with decreased 5-HTT expression actually increasing resilience to adulthood stress. Furthermore there is anthropological evidence that decreased 5-HTT expression is protective against infection and depression, and immunological evidence that high 5-HT levels protect against pro-inflammatory responses and depression. To our knowledge this is the first animal-model study of these complex G→endophenotype and GxE→state marker inter-relationships. Evidence is presented that, relative to WT littermates, adult 5-HTT KO HET mice exhibit endophenotypes of increased basal 5-HT and DA and respective major metabolites in nucleus accumbens and increased basal plasma corticosterone. Furthermore, adult 5-HTT KO HET mice exposed to psychosocial stress exhibit, relative to WT littermates, resilience markers in the form of an absence of increased plasma TNF, absence of spleonomegaly, decreased basal plasma corticosterone, and an absence of increased fear conditioning. As such, the current findings provide important new insights into how reduced 5-HTT expression can increase stress resilience, with high 5-HT levels acting to reduce the pro-inflammatory response to stress appearing to be a major factor in this complex network.

**Endophenotypes of 5-HTT genotype**

Adult male control mice that were heterozygous knockout for the 5-HTT gene demonstrated increased tissue levels of 5-HT and its major metabolites 5-HIAA and 3-MT in the NAcc, relative to WT. This finding is consistent with increases in each of tonic 5-HT release, reuptake and metabolism at 5-HT terminals in the NAcc of HET mice. The lack of genotype effect on 5-HT turnover suggests that there would not be a major effect on 5-HT synaptic levels. These findings are clearly not explicable in terms of a simple reduction in 5-HTT reuptake of 5-HT and suggest that the effects of reduced 5-HTT on 5-HT neurochemistry are complex. In a microdialysis study, HET mice had increased basal 5-HT release in both regions studied, namely dorsal striatum and frontal cortex, relative to WT (Mathews et al., 2004). In an *ex vivo* study of tissue levels in frontal cortex, there was no genotype effect on absolute 5-HT levels or turnover (Bartolomucci et al., 2010). In the present study, HET mice also demonstrated increased NAcc tissue levels of DA and its major metabolites DOPAC and HVA relative to WT. This further indicates the complex effects of 5-HTT genotype-phenotype on monoamine neurochemistry. As for 5-HT, the DA findings are consistent with increases in tonic DA release, reuptake and metabolism, and the turnover values suggest that DA synaptic levels are increased in HET relative to WT mice. Clearly it will be important to measure effects of 5-HTT genotype on monoamine levels in other brain regions and in blood.

Control-HET mice exhibited a further endophenotype in the form of increased basal plasma corticosterone relative to WT. A previous study reports no HET versus WT difference for this parameter (Bartolomucci et al., 2010). In healthy humans, 5-HTTLPR S-allele carriers exhibited increased salivary waking cortisol levels relative to L-allele carriers (O'Hara and Hallmayer, 2007), indicating an interesting similarity to the current data. One of the major functions of glucocorticoids is

their antagonism of pro-inflammatory processes (Rhen and Cidlowski, 2005). Whilst this anti-inflammatory effect has been most studied in terms of pathogen-induced inflammation, it has also been put forward in the context of psychosocial stress-induced inflammation (Miller et al., 2009). Therefore, the endophenotype of increased blood levels of corticosterone might contribute to the absence of immuno-inflammatory responses to CSD that was observed in HET mice (see below).

In terms of behavioural measures, there was no evidence for 5-HTT-related endophenotypes. To our knowledge there is no previous study of acquisition of CS fear conditioning in HET versus WT 5-HTT KO mice. A study of expression and extinction of tone CS-conditioned fear reports an absence of genotype effects on both expression and extinction (Narayanan et al., 2011). In other behavioural tests or paradigms of emotionality, there was no HET-WT difference in an elevated plus maze test of anxiety and an anxiogenic endopheotype of HET mice in terms of increased latency to enter and decreased time spent in the light compartment in a dark-light box test (Jansen et al., 2010). In human, the S allele of the 5-HTTLPR polymorphism is associated with increased neuroticism and anxiety in healthy probands (Lesch 1996). Furthermore, it is associated with increased fMRI activation of amygdala and hippocampus in response to emotionally negative facial expressions, e.g. fear (Canli et al., 2006; Hariri et al., 2002).

**5-HTT-genotype modulation of the effects of chronic social defeat**

In the wildtype mice, the effects of psychosocial emotional stress induced by CSD were robust and directly in line with those obtained previously (see Chapter 2). Therefore, CSD led to increased day-to-day body weight variation, elevated plasma TNF level and splenomegaly, and increased emotional reactivity to an aversive stimulus in the form of increased fear conditioned freezing. In a previous study of the effects of CSD in WT mice of a 5-HTT KO strain, CSD led to increased absolute body weight and increased plasma basal corticosterone relative to WT-CON mice (Bartolomucci et al., 2010); both of these effects are in contrast to the present findings. At the behavioural level, CSD led to increased social avoidance in WT mice, suggesting increased expression of fear to the social stimuli associated with the unconditioned stimulus of social aggression/defeat.

Each of the stress state markers demonstrated by WT-CSD mice is relevant to state markers of depression. Therefore, loss or gain of body weight are depression symptoms (DSM-5, 2013; ICD-10, 1994) and low self-esteem and negative affect are associated with increased body weight variation (Foreyt et al., 1995; Serdar et al., 2011). Psychosocial stress increases blood levels of pro-inflammatory cytokines (Kiecolt-Glaser et al., 2005; McDade et al., 2006) and a meta-analysis found that TNF is increased in depressive patients (Dowlati et al., 2010). In depression there is increased aversive fear conditioning (Nissen et al., 2010). The lack of effect of CSD on basal plasma corticosterone in WT mice is somewhat inconsistent with human depression, in which 40-60% of patients exhibit increased basal cortisol levels relative to healthy probands (Parker et al., 2003). With respect to aetio-pathophysiology, the inflammation hypothesis of depression proposes that psychosocial stress-induced immune-inflammation in the periphery activates central inflammatory processes (Miller et al., 2009). Furthermore, the negative impact of immuno-inflammation on monoamine neurotransmission – both 5-HT and DA – has been proposed as a major aetio-

pathophysiological pathway for depression (Felger and Miller, 2012; Haroon et al., 2012). The current findings, and in particular a companion study (Chapter 2), provide strong support for this hypothesis.

Interestingly, in comparison with WT, the effects of CSD were attenuated in their 5-HTT KO HET littermates. Therefore, there was no consistent effect of CSD on day-to-day body weight variation, plasma TNF level, spleen weight, and fear conditioned freezing. This relative resilience to CSD in HET mice cannot be attributed to basal HET-WT differences because HET mice did not exhibit an endophenotype on any of these parameters. The parameters on which HET-CON mice did exhibit an endophenotype relative to WT-CON were basal NAcc 5-HT and DA and their metabolites, which were increased in HET-CON (and HET-CSD), and basal plasma corticosterone, which was also increased in HET-CON. Given the evidence that 5-HT exerts important inhibitory effects on pro-inflammatory cytokines (Kubera et al., 2005), then the increase in 5-HT activity could be protective in HET mice against stress-induced inflammation. Given the evidence that corticosterone exerts important antagonistic effects on pro-inflammatory processes (Rhen and Cidlowski, 2005), then the high basal level could be protective in HET mice against stress-induced inflammation. Also, with respect to the hypothesis that immuno-inflammation reduces 5-HT and DA neurotransmission, then the high tissue levels of these monoamines in NAcc (and possibly other regions) exhibited by HET mice could be protective. Indeed, in the companion CSD study, CSD induced de-regulation in expression of genes involved in DA signalling in AMYG in C57BL/6 mice (Chapter 2). It has been reported that, relative to WT-CSD mice, HET-CSD mice exhibit increased avoidance of CD-1 mice indicating, as noted above, increased reactivity to a specific aversive stimulus. Similarly, we have demonstrated that HET mice are more sensitive than WT mice to specific learned helplessness, using electroshock during both pre-exposure and test (Pryce et al., 2012). This increased reactivity to specific social or physical stimuli experienced as aversive is adaptive, suggesting that reduced 5-HTT function both increases adaptive aversive learning and decreases generalisation across stimuli which is indeed maladaptive (Chapter 2).

As noted in the Introduction of this Chapter, there is meta-analytical evidence that reduced 5-HTT expression/function increases resilience to life stress events in adulthood, as measured in terms of depression prevalence (Karg et al., 2011). The current mouse-model study provides evidence for a protective effect of reduced 5-HTT in terms of preventing the increase in emotional reactivity to aversive physical stimuli induced by chronic psychosocial stress in adulthood. Furthermore, it provides novel evidence that increased central 5-HT and DA basal activity, increased plasma corticosterone and, possibly related to these endopheotypes, absence of stress-induced immuno-inflammation, underlie the increased emotional resilience.

# 6. ACKNOWLEDGEMENTS

This research was funded by the Swiss National Science Foundation grants 31003A-130499 and 31003A-141137.

# Chapter 4

**Dissociation of effects of RS- and S-ketamine at low and high doses on mouse antidepressant-test behaviour, prefrontal-cortical glutamate release and BDNF and mTOR levels**

Damiano Azzinnari1, 4, Hannes Sigrist1, Kilian Jörg1, Boris Ferger2, Erich Seifritz3, 4, Christopher R. Pryce1, 4

1 Preclinical Laboratory for Translational Research into Affective Disorders, Department of Psychiatry, Psychotherapy and Psychosomatics, Psychiatric Hospital, University of Zurich, Switzerland

2 CNS Diseases Research, Boehringer Ingelheim Pharma GmbH & Co. KG., Biberach, Germany

3 Department of Psychiatry, Psychotherapy and Psychosomatics, Psychiatric Hospital, University of Zurich, Switzerland

4 Neuroscience Center Zurich, University of Zurich and ETH Zurich, Switzerland

# 1. SUMMARY

Ketamine is of marked interest in psychiatry in various contexts, most recently as a low-dose, acute experimental therapy for treatment-resistant depression. The latter has stimulated rodent studies of low-dose ketamine aimed at elucidating antidepressant mechanism-of-action. Fundamental issues require clarification. *Enantiomer*: nearly all human and rodent studies have used the less-specific and -potent RS-ketamine and not S-ketamine. *Glutamate release*: only medium-high dose RS-ketamine has been studied. *Signalling pathway*: studies of putative major proteins including brain-derived neurotrophic factor (BDNF) and mammalian target of rapamycin (mTOR) have used RS-ketamine specifically. In the present mouse experiments the effects of acute RS- and S-ketamine at equivalent low (3.0, 1.5 mg/kg) and high (50, 25 mg/kg) doses were compared in terms of effects on: floating in a desipramine-validated forced swim test (FST), escape in an imipramine-validated learned helplessness (LH) paradigm, glutamate release in medial prefrontal cortex (mPFC), BDNF levels in hippocampus (HIPP), and mTOR levels in HIPP and mPFC. Floating in FST was decreased by RS-ketamine at 3.0 mg/kg specifically; escapes in the LH paradigm were unaffected by all ketamine treatments; mPFC glutamate release was increased by RS- at 50 mg/kg and S-ketamine at 25 mg/kg, specifically; HIPP BDNF was increased by 1.5 mg/kg S-ketamine specifically; HIPP and mPFC mTOR were unaffected by all ketamine treatments. This study presents novel and important evidence that the acute ketamine effect in the FST antidepressant screening test is low-dose RS-ketamine-specific, and that it occurs independently of increased mPFC glutamate release, increased HIPP BDNF and increased HIPP and mPFC mTOR.

# 2. INTRODUCTION

Depression is a prevalent neuropsychiatric disorder and has one of the highest overall burdens across all disease classes (Wittchen et al., 2011). Treatment resistance and relapse are common (Little, 2009) and novel efficacious treatments are needed. Several small clinical studies report rapid-onset antidepressant efficacy of ketamine in treatment-resistant depression (Berman et al., 2000; Zarate et al., 2006). Ketamine is an antagonist at the NMDA glutamate receptor by blocking the phencyclidine binding site (Mion and Villevieille, 2013; Vollenweider et al., 1997). Ketamine is used as an anaesthetic despite psychotomimetic side-effects. The psychotomimetic effects are of interest to psychiatry as a means of increasing psychotherapy responsiveness (Vollenweider and Kometer, 2010); they also account for ketamine being a drug of abuse (De Luca et al., 2012). Medium-high (sub-anaesthetic) doses of ketamine have been applied in rodents to induce neurobehavioural states relevant to positive, cognitive and negative schizophrenia symptoms (Brody et al., 2003; Neill et al., 2010; Ong et al., 2005). To-date, most translational psychiatry research has used RS-ketamine. This is despite S-ketamine having a 4- and 2-fold higher affinity for the NMDA phencyclidine binding site relative to R- and RS-ketamine, respectively (Mion and Villevieille, 2013; Vollenweider et al., 1997). RS-Ketamine also acts at sigma and opioid receptors (Hustveit et al., 1995; Narita et al., 2001; Robson et al., 2012); the relative affinities of the ketamine enantiomers for these additional binding sites are currently unknown.

Ketamine studies in treatment-resistant depression have almost exclusively used a single low-dose infusion of RS-ketamine, e.g. 0.5 mg/kg during 40 min (Zarate et al., 2006). Rodent studies of ketamine induction of schizophrenia-relevant behavioural states use medium-high dose RS-ketamine (rat: 10-30 mg/kg, mouse: 100 mg/kg) (Brody et al., 2003; Neill et al., 2010; Ong et al., 2005). In rat, this regimen stimulates glutamate release in the medial prefrontal cortex (mPFC) (Lorrain et al., 2003; Moghaddam et al., 1997). The recent reports of ketamine therapy for treatment-resistant depression have stimulated rodent studies of ketamine effects in antidepressant screening tests (e.g. forced swim test (FST)) and models of depression (e.g. chronic unpredictable mild stress-induced loss of sucrose preference). These studies have also used RS-ketamine and primarily at low-medium doses, consistent with the low antidepressant dose in human studies and the importance of distinguishing from the schizophrenia animal models. Findings are heterogeneous: For example, in the FST in mouse and rat there are reports of a lack of effect of acute RS-ketamine at low doses, with effects only emerging at medium-high doses (Cruz et al., 2009; Reus et al., 2011). However, there are prominent reports that acute low-dose RS-ketamine (2.5-5.0 mg/kg) induces increased activity (decreased floating) in the FST in mice (e.g. (Autry et al., 2011)), analogous to the effects of selective monoamine reuptake inhibitor antidepressants (Cryan et al., 2002), and increased escape responses in a mouse learned helplessness (LH) paradigm (Maeng et al., 2008). In rat, acute medium-dose (10 mg/kg) RS-ketamine induced increased activity in the FST (Li et al., 2010), an effect analogous to that of antidepressants (Porsolt et al., 1977), and 10 mg/kg RS-ketamine reversed the loss of sucrose preference induced by chronic unpredictable mild stress in rats (Li et al., 2011).

Regarding an antidepressant mechanism-of-action of low-dose ketamine, the triggering effects of NMDA-receptor blocking are considered to be two-fold: (1) Increases in binding of glutamate and signaling by AMPA receptors (AMPA-R) on post-synaptic pyramidal neurons in the PFC. (2) Inhibition of GABA interneurons acting at glutamate projection neurons, resulting in disinhibition of and increased glutamate release by the latter into synapses with PFC pyramidal neurons (Autry et al., 2011; Duman et al., 2012; Vollenweider and Kometer, 2010). Regarding point (2), it is important that the evidence for increased PFC glutamate release was obtained in rat using RS-ketamine at medium-high doses (10-30 mg/kg: (Lorrain et al., 2003; Moghaddam et al., 1997)) and therefore mainly exceeding those used to demonstrate low-dose RS-ketamine effects in rodent screening tests (e.g. (Autry et al., 2011)) and depression models (e.g. (Li et al., 2011)). Increased AMPA-R signaling has been proposed to activate the pathway leading to increased translation of brain-derived neurotrophic factor (BDNF), occurring in hippocampus (HIPP) within 30 minutes of low-dose RS-ketamine (Autry et al., 2011). BDNF release leads to local activation of TrkB receptors and the pathway leading to phosphorylation of mammalian target of rapamycin (mTOR). Phosphorylated mTOR is a major and rapid regulator of synaptic protein synthesis and its level in mPFC increases within 30 minutes of low-medium-dose RS-ketamine (Li et al., 2010). Increased BDNF release and mTOR phosphorylation has been proposed as the proximate mechanism underlying low-dose ketamine antidepressant action (Duman et al., 2012).

In the present study, important issues raised by this translational evidence for acute low-dose ketamine as an antidepressant treatment were addressed. In C57BL/6 mice, the following effects were investigated: (1) RS- or S-ketamine at equivalent low (3, 1.5 mg/kg) or high (50, 25 mg/kg) dose in the FST. (2) RS- or S-ketamine at equivalent low dose in a LH paradigm. (3) RS- or S-ketamine at equivalent low or high dose on glutamate release in mPFC. (4) RS- or S-ketamine at equivalent low or high dose on BDNF in HIPP. (5) RS- or S-ketamine at equivalent low or high dose on mTOR in HIPP and mPFC. Of particular interest was whether a low-dose of high-affinity S-ketamine exerted the most robust effect in antidepressant screening tests and, if so, whether this was associated with increased mPFC glutamate release, increased BDNF levels and increased mTOR phosphorylation.

# 3. EXPERIMENTAL PROCEDURES

## 3.1 Animals and maintenance

Male C57BL/6J mice were obtained from Janvier (Le Genest Saint Isle, France). At study onset mice weighed 22.0-30.0 g. They were maintained on a reversed 12:12 h light-dark cycle (lights off 07:00-19:00 h) in an individually-ventilated caging system (IVC) at 20-22 °C and 50-60% humidity. Cages were type 2L and contained woodchips, a sleep igloo and tissue bedding. Complete-pellet diet (Provimi, Kliba Ltd, Kaiseraugst, Switzerland) and water were available continuously and *ad libitum*. The study was conducted under a permit (110/2009) for animal experimentation issued by the Veterinary Office of Zurich, Switzerland. All efforts were made to minimize the number of mice used and any unnecessary stress.

## 3.2 Drugs and administration

RS-ketamine (Ketasol-100, Graeub, Bern, Switzerland) and S-ketamine (Keta-S, Graeub) were used; with respect to relative NMDA-Phencyclidine affinities a ratio of 1:2 was assumed (Mion and Villevieille, 2013; Vollenweider et al., 1997). In each experiment the following treatments were included: 0.9% saline (SAL), RS-ketamine at 3 and 50 mg/kg (except LH paradigm, 3 mg/kg only), S-ketamine at 1.5 or 25 mg/kg (except LH paradigm, 1.5 mg/kg only), and in some experiments S-ketamine also at 7.5 mg/kg. In the FST, desipramine (DES, desipramine hydrochloride, Sigma-Aldrich, St. Louis (MO), USA) at 10 mg/kg was used as a positive control. In the LH paradigm, imipramine (IMI, imipramine hydrochloride, Sigma-Aldrich) at 20 mg/kg was used as a positive control. All drugs were administered in a volume of 5 ml/kg and concentrations refer to the base.

## 3.3 Effects of ketamine on behaviour in FST and LH paradigm

**Forced swim test**

Mice aged 9-12 weeks were singly caged, and maintained in the experimental room and handled on the 3 days prior to the experiment. The FST was conducted using a 5 L Plexiglas cylinder (∅ = 18 cm, height = 25 cm) filled with 4 L clean water at 23-24°C. The sample size was 8-12 mice per drug group. Mice were injected acutely i.p. with ketamine, SAL or DES. Two separate experiments were conducted at 10:00-13:00 h, one investigating acute drug effects at 30 min and the other at 180 min. The mouse was placed gently in the water and behaviour was scored by two independent observers who were blind to drug group. The instantaneous sampling method of behavioural scoring was used with an interval of 10 sec, such that at every $10^{th}$ second the on-going behaviour from the following mutually-exclusive list was given a score of 1: climbing: scratching on the cylinder with both of the forelimbs; swimming: cycling movement of the entire hind limbs; paddling: one or both hind paws is/are extended and flexed; floating: either no movement of hind limb(s) or maximally one hind limb slowly extended and flexed for balancing. The duration of the test was 6 min per mouse. The mouse was then removed, dried and warmed under a heating lamp and returned to its home cage. The test measure was the total score for floating during min 3-6 inclusive (maximum score = 4 x 6 = 24). Inter-observer

reliability, expressed as the Pearson correlation for observers' floating scores for 20 tests selected at random, was r = +0.98.

**Learned helplessness**

Mice aged 10-14 weeks were housed in littermate pairs and handled on the 3 days prior to the experiment. The LH paradigm was conducted using a multi-purpose aversive conditioning apparatus (Multi Conditioning System, TSE Systems GmbH, Bad Homburg Germany (Pryce et al., 2012)). Briefly, a quadratic arena (30 x 30 x 24 (H) cm) was placed on an electrified grid floor and was divided into equal left and right compartments by a central divider raised off the floor so that the mouse could transfer between compartments by passing underneath it. All procedures were conducted at 13:00-16.00 h. The sample size was 4-9 mice per group. The protocol was as follows: Day 1, arena habituation: 15 min session without electroshocks. Day 2, pre-exposure to escapable electroshocks (ES): 30 x 0.2 mA x 5 sec ES at 50 sec intervals. Days 3-4, pre-exposure to inescapable electroshocks (IS): 24 x 0.15 mA x 1-5 sec IS at 50 sec intervals. Day 5, screening escape test: 24 x 0.15 mA x 5 sec ES at 50 sec intervals. Mice were allocated to drug groups by counter-balancing according to number of escape responses at day 5. Day 6, escape test: Mice were injected acutely i.p. with ketamine or SAL at 180 min pre-test, and test conditions were 30 x 0.15 mA x 5 sec ES at 50 sec intervals. For the positive control experiment, IMI or SAL were injected acutely i.p. at 30 min pre-test. The test measure was frequency of escape failure in each of three 10-trial blocks.

## 3.4 Effects of ketamine on mPFC glutamate release

Mice were 11–16 weeks at study onset. For brain cannulation, mice were anaesthetized (i.p cocktail: fentanyl 0.04 mg/ kg, midazolam 4 mg/kg, medetomidin 0.4 mg/kg). The head of the mouse was fixed in a stereotaxic frame and an intracerebral guide cannula (MAB 4.6. IC, Microbiotech, Stockholm, Sweden) was placed above the mPFC of the right hemisphere using the following coordinates relative to bregma: AP: +1.7 mm, ML: +0.2 mm (Franklin and Paxinos, 2008) with modification based on pilot study for the mouse strain used). The cannula was fixed onto the skull with dental cement (Paladur, Heraeus, Hanau, Germany) using two stainless steel screws for additional fixation. Post-surgery, mice were singly caged for a 6-13 day recovery period.

The evening before microdialysate collection, mice were anaesthetised briefly using sevoflurane and a microdialysis probe with a membrane of 1 mm length and a shaft length set so that the membrane tip extended DV -3.0 mm (from skull) (MAB 4.6.1. Cuprophane, Microbiotech) was inserted into the guide cannula. The mouse was placed into a 20 L Plexiglas cylinder containing food and water. The cannula inlet/outlet were connected via fine tubing to the pump and dual-channel swivel. Artificial cerebrospinal fluid (aCSF, M: KCl 2.7, NaCl 147.0, $CaCl_2$ 1.2, $MgCl_2$ 0.85, $NaH_2PO_4$ 1.0, pH 7.4) was pumped at 1 µl/min for overnight equilibration. Beginning at 08:00 h, four baseline samples were collected at 20 min (20 µl) intervals. The mouse was held, injected s.c. and returned to the microdialysis cylinder. Sampling at 20 min intervals was conducted for 120 min. Samples were frozen on dry ice and stored at -80 °C. The mouse was then deeply anaesthetized and aCSF was replaced with 1% methylene blue-$H_2O$ for 10 min infusion to facilitate determination of probe location. The brain was fixed, then frozen and cut at 100 µm on a microtome for localization of the methylene blue spot. The total sample was 9-15 mice per drug group and following exclusion due to either

missing dialysate samples or probe mis-location, the final sample size was 6-11 complete sample sets per drug group. For mice accepted into the data set the average location of the probe was AP +1.62 mm (range 1.34-1.94 mm, Fig. 3D). As a positive control for glutamate release, in several mice, after completion of drug-sample collection, the aCSF was replaced with potassium-enriched aCSF (mM: NaCl 99.7, KCl 50.0, $CaCl_2$ 1.2, $MgCl_2$ 0.85 and $Na_2HPO_4$ 1.0, pH 7.0–7.4) for 20 min and microdialysates were collected at 20-min intervals for a further 120 min (see (Buck et al., 2009)).

Glutamate concentration in microdialysates was measured using high performance liquid chromatography (HPLC) coupled with fluorescence detection (HPLC–FD), using a method based on that described for glycine (Voehringer et al., 2013). Glutamate standards were prepared using L-glutamic acid (G1251, Sigma-Aldrich) in Millipore $H_2O$ (1-10000 nM). Microdialysate (5 µl) was diluted with 20 µl Millipore $H_2O$, and 15 µl were combined with 3 µl OPA-reagent (0.5% 2-mercaptoethanol (4227.3, Carl Roth, Karlsruhe, Germany) in phthaldialdehyte reagent (P7914, Sigma-Aldrich) and injected onto the HPLC-FD column. Mobile phase A consisted of 4625 ml 0.1 M sodium acetate, 250 ml methanol and 125 ml tetrahydrofuran, and mobile phase B of 97.5 % methanol and 2.5 % tetrahydrofuran. The gradient elution profile was as follows at a constant flow rate of 1.2 ml/min: 0.0 min 100 % A → 3.0 min 100 % A → 4.0 min 100 % B → 6.0 min 100 % B → 7.0 min 100 % A → 8.5 min 100 % A. For each mouse, post-drug microdialysate glutamate concentrations were expressed as a percentage of its mean baseline concentration. Reported values are non-corrected for *in vitro* estimated recovery (15 %).

## 3.5 Effects of ketamine on region-specific BDNF and mTOR

**Brain tissue preparation**

Mice were aged 10 weeks and singly caged. They were injected i.p. at 07:00-08:00 h, returned to the home cage and decapitated after 30 min. Brains were removed and fresh fixed on dry ice. Frozen brains were sectioned coronally at 1.0 mm intervals using a stainless steel brain matrix (model MMCS-1, Plastics One, Roanoke VA, USA) and single-edge blades (model 10-100-063, Apollo Herkenrath, Solingen, Germany). Regions of interest, namely dorsal (d) HIPP and mPFC (infralimbic cortex + prelimbic cortex) were microdissected bilaterally from the corresponding sections using a brain punch (∅ = 1.00 mm, model 57397, Stoelting Europe, Dublin, Ireland) and a mouse brain atlas (Franklin & Paxinos, 2008): dHIPP (2 biopsies/hemisphere) at bregma -1.5 to -2.4 ± 0.2 mm and mPFC (1 biopsy/hemisphere) at bregma 2.2 to 1.3 ± 0.2 mm. Tissue mass was 0.6-1.0 mg per biopsy. All microdissection steps were conducted at -18 °C. Brain biopsies were stored in 1.5 ml Protein LoBind tubes (Eppendorf, Hamburg, Germany) at -80 °C. Samples were sonicated (VibraCell, VCX130PB, Sonics and Materials, Newtown CT, USA) in 100 µl lysis buffer: 100 mM NaCl, 1 mM EGTA, 20 mM Tris (pH 7.5), 1 mM EDTA, 1% Triton-X-100, 1% Protease inhibitor solution (Thermo Fisher Scientific, Waltham MA, USA), 1% Phophatase inhibitor I (Sigma-Aldrich), 1% Phosphatase inhibitor II (Sigma-Aldrich). After centrifugation at 20,000-g for 10 min at 4°C the supernatant was pipetted into a LoBind tube and stored on ice. Total protein concentration was quantified using the Coomassie Plus Bradford assay kit (Thermo Fisher Scientific) according to manufacturer's instructions with minor modifications. The assay was run in a 96-well microtitre plate (Nunc, Roskilde, Denmark) and the albumin standard

(25-750 µg protein/ml) was run in triplicate using 10 µl standard and 2 µl lysis buffer per well. Lysates were measured in duplicate using 2 µl and 10 µl $H_2O$ per well. Coomassie Plus protein assay reagent was added at 250 µl per well and optical density was measured at 650 nm (SpectraMax M2, Molecular Devices, Sunnyvale CA, USA).

**BDNF immunoassay**

Brain-derived neurotrophic factor was measured in dHIPP lysates using an enzyme-immunoassay kit (BDNF Emax ImmunoAssay system, Promega, Madison, USA). The assay was conducted according to the manufacturer's instructions, with lysates (100 µl/sample) prepared without acid-treatment and diluted 1:10 in block and sample buffer, and measured in duplicate within a single microtitre plate. BDNF concentrations were normalized to protein concentrations (pg BDNF/mg protein). For mPFC, BDNF levels were below the limit of detection (3 pg/ml).

**Phosphophorylated and total mTOR immunoassay**

Phosphorylated and total mammalian target of rapamycin were measured in mPFC and dHIPP lysates using a 2-spot electrochemiluminescence immunoassay kit (Phospho(Ser2448)/Total mTOR Assay Whole Cell Lysate Kit, Meso Scale Discovery, Rockville, USA). The assay was conducted according to the manufacturer's instructions. Per sample, a volume containing 20 µg protein (5-30 µl) was added in duplicate to wells of the microtitre plate and the volume adjusted to 30 µl with lysis buffer. The plate was read using a SECTOR® Imager 2400 (Meso Scale Discovery). Electrochemiluminescence signals for phospho- and total-mTOR for all drug groups were expressed relative to the mean total mTOR signal of the SAL group. It is important to note that in the present study and in previous studies from other laboratories (e.g. (Bradtmoller et al., 2012)), for all samples, the estimate of phospho-mTOR was greater than that of total-mTOR; the kit manufacturer attributes this to the increased affinity for phospho-mTOR of the specific phospho-mTOR antibody relative to the total-mTOR antibody as used in the kit (MSD, personal communication).

## 3.6 Statistical analysis

Statistical analysis of ketamine effects on antidepressant screening-test behavior, glutamate release and region-specific signaling protein concentrations was conducted using SPSS (version 20, SPSS Inc., Chicago IL, USA). In most cases ANOVA was used, with a between-subject factor of drug dose and, depending on parameter, a within-subject factor (e.g. trial-block in LH paradigm, sampling interval in microdialysis experiment). *Post hoc* testing was conducted using Bonferroni correction for multiple comparisons. Statistical significance was set at $p < 0.05$. Where an estimate of variance is given this is the standard deviation (SD).

# 4. RESULTS

## 4.1 Forced swim test

Relative to SAL, DES, a positive control, led to decreased floating at the 30-min ($t_{(18)}$ = 6.20, $p$ < 0.0005, Fig. 1A) and not at the 180-min test ($p$ = 0.52, Fig. 1B). For RS-ketamine, at 30 min there was a borderline non-significant effect of dose on floating ($F$(2, 24) = 3.2, $p$ < 0.06, Fig. 1C), with 3 mg/kg associated with a mean decrease relative to SAL. At 180 min, there was a main effect of dose ($F$(2, 25) = 6.76, $p$ < 0.005, Fig. 1D): 3 mg/kg RS-ketamine decreased floating relative to SAL ($p$ < 0.003) and 50 mg/kg was without effect. For S-ketamine, at 30 min there was a borderline non-significant effect of dose ($F$(2, 25) = 3.3, $p$ < 0.06, Fig. 1E), with 1.5 mg/kg associated with a mean decrease relative to SAL. At 180 min, there was no effect of S-ketamine at 1.5, 7.5 or 25 mg/kg relative to SAL ($p$ = 0.80, Fig. 1F).

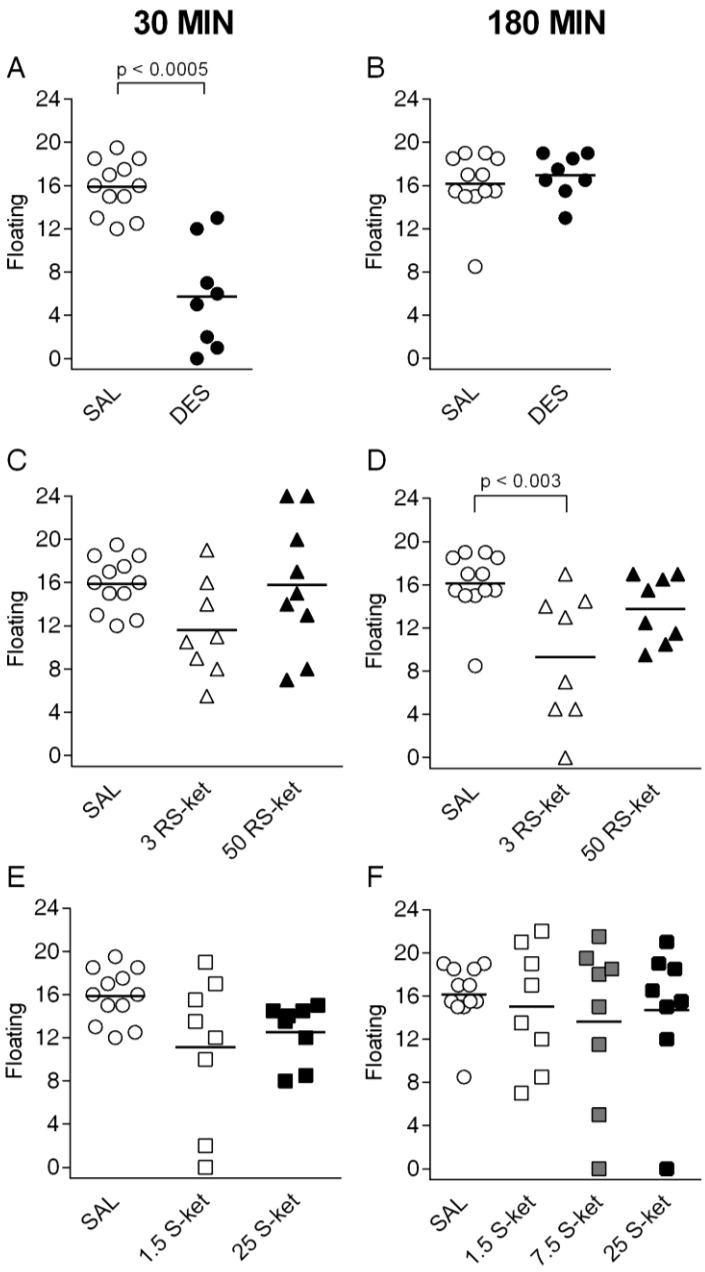



Figure 1. Scatter plots depicting effects of acute i.p. treatment with desipramine (DES), RS- or S-ketamine (-ket) on mouse floating behavior in the forced swim test (FST). (A-B). DES (10 mg/kg) positive control versus saline (SAL) at (A) 30-min test or (B) 180-min test. Sample size was 8-12 mice per dose/experiment. (C-D). RS-Ket (3, 50 mg/kg) at (C) 30-min test or (D) 180-min test. Sample size was 8-12 mice per dose/experiment. (E-F) S-Ket (1.5, 7.5, 25 mg/kg) at (E) 30-min test or (F) 180.min test. Sample size was 8-12 mice per dose/experiment. Black bar denotes the mean.

## 4.2 Learned helplessness paradigm

With IMI, used as a positive control, there was a significant dose x trial-block interaction in the escape test ($F(2, 22) = 7.5$, $p < 0.003$, Fig. 2A): *post hoc* analysis indicated decreased escape failure by IMI mice relative to SAL mice in trials 11-20 ($p < 0.05$) and 21-30 ($p < 0.02$). For low-dose RS- and S-ketamine there was no effect on escape failure in the escape test ($p = 0.77$, Fig. 2B).

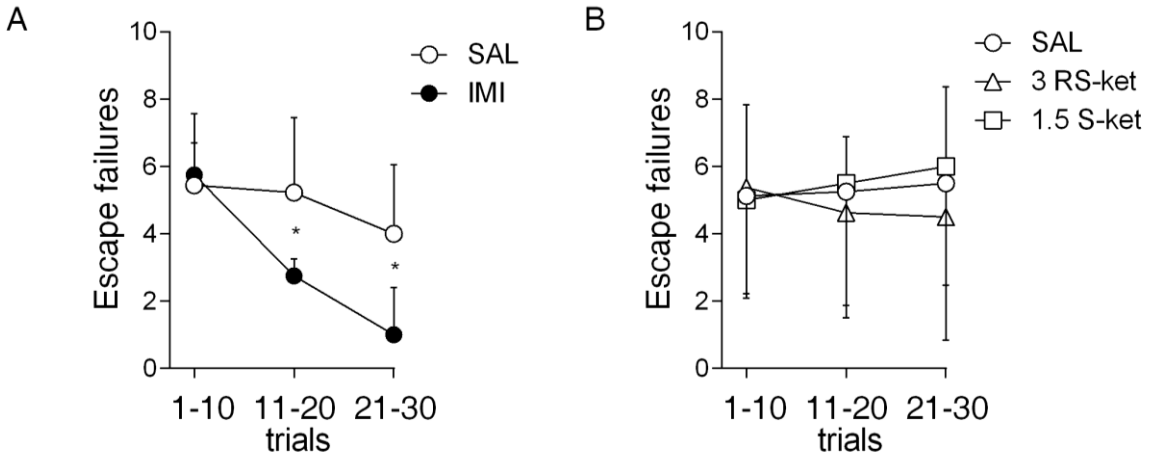



Figure 2. Effects of acute i.p. treatment with imipramine (IMI), RS- or S-ketamine (-ket) on escape behavior in the two-way escape test. Mice were pre-exposed to inescapable electroshock on two days and were allocated to dose groups by counter-balancing according to escape failures in a screening escape test on the previous day. The escape test consisted of 30 trials of escapable electroshocks (maximum 5 sec) that were analysed as three 10-trial blocks. (A) IMI (20 mg/kg, N=4) positive control versus saline (SAL, N=9) at 30 min before the escape test. (B) RS-ket (3 mg/kg, 3 RS-ket, N=8) and S-ket (1.5 mg/kg, 1.5 S-ket, N=6) versus SAL (N=8) at 180 min before the escape test. Values are mean +/- standard deviation. * $p < 0.05$ in trials 11-20 and 21-30.

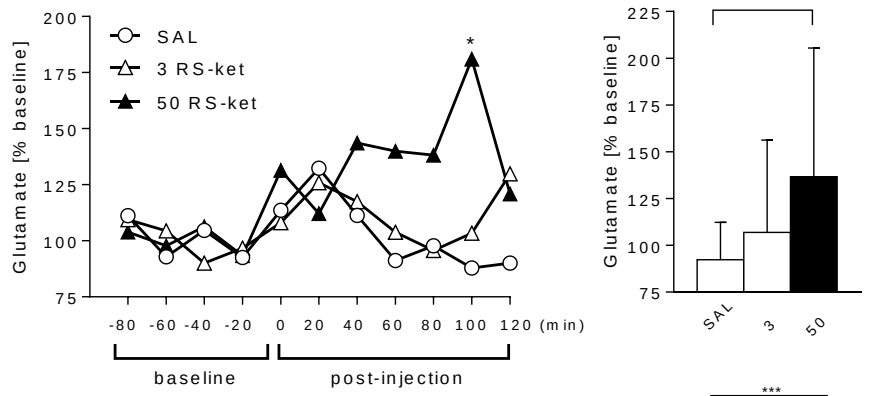
## 4.3 mPFC glutamate release

For RS-ketamine, mean absolute basal glutamate concentrations were similar across drug groups: SAL 401±176 (nM), 3 mg/kg 511±154, 50 mg/kg 538±143 ($p = 0.30$). There was a main effect of dose on mPFC glutamate concentrations relative to baseline ($F(2, 20) = 3.4$, $p < 0.05$) and no effect of sampling interval ($p = 0.87$) (Fig. 3A): *post hoc* analysis indicated that 50 mg/kg increased glutamate release relative to SAL ($p < 0.05$) whereas 3 mg/kg was without effect ($p = 0.92$). The maximum increase in glutamate release induced by 50 mg/kg was observed at min 100 post-injection (Fig. 3A).

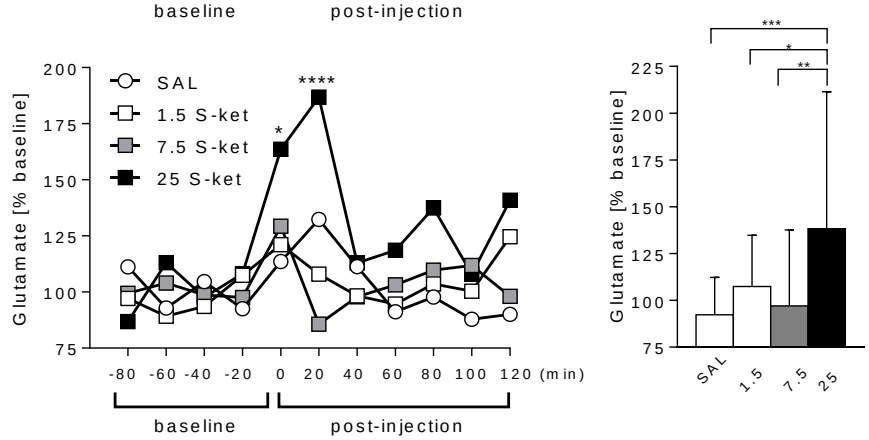
For S-ketamine, mean absolute basal glutamate concentrations were similar across drug groups: SAL 401±176 (nM), 1.5 mg/kg 364±128, 7.5 mg/kg 534±390, 25 mg/kg 329±156 ($p = 0.41$). There was a main effect of dose on mPFC glutamate concentrations relative to baseline ($F(3, 32) = 6.8$, $p < 0.001$) and a main effect of sampling interval ($F(6, 187) = 2.4$, $p < 0.03$) (Fig. 3B). Regarding dose, *post hoc* analysis indicated that 25 mg/kg increased glutamate release relative to SAL ($p < 0.001$), 1.5 mg/kg ($p < 0.05$) and 7.5 mg/kg ($p < 0.003$). The maximum increase in glutamate release induced by 25 mg/kg was observed at min 20 post-injection (Fig. 3B). Regarding time, *post hoc* analysis indicated that no two sampling intervals differed significantly in terms of overall mean glutamate release.

Potassium-enriched aCSF provided a positive control, as indicated by the main effect of sampling interval ($F(8, 32) = 5.9$, $p < 0.0005$, Fig 3C): *post hoc* analysis indicated increased glutamate release relative to baseline at min 40 post-injection ($p < 0.003$).

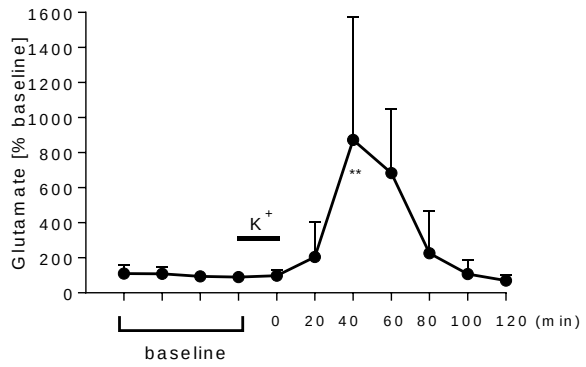
A


SAL
3 RS-ket
50 RS-ket
Glutamate [% baseline]
200
175
150
125
100
75
*
-80 -60 -40 -20 0 20 40 60 80 100 120 (min)
baseline
post-injection
225
200
175
150
125
100
75
*
SAL
3
50

B


SAL
1.5 S-ket
7.5 S-ket
25 S-ket
Glutamate [% baseline]
200
175
150
125
100
75
****
*
-80 -60 -40 -20 0 20 40 60 80 100 120 (min)
baseline
post-injection
225
200
175
150
125
100
75
***
*
**
SAL
1.5
7.5
25

C


Glutamate [% baseline]
1600
1400
1200
1000
800
600
400
200
0
K+
**
0 20 40 60 80 100 120 (min)
baseline

D

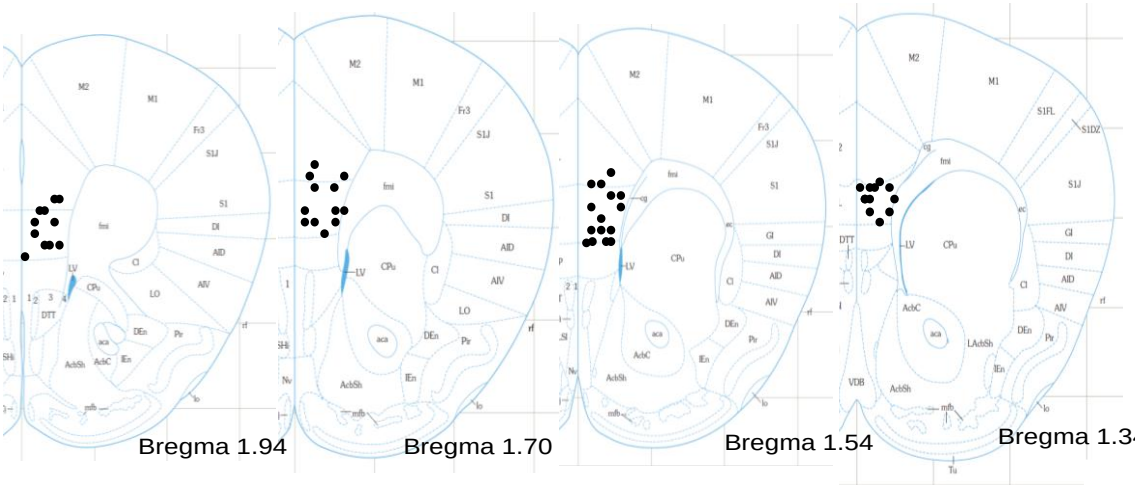

Bregma 1.94
Bregma 1.70
Bregma 1.54
Bregma 1.34

Figure 3. Effects of acute s.c. RS- or S-ketamine (-ket) on glutamate release (concentration as % of mean baseline concentration) in the mouse medial prefrontal cortex. (A) Time course for RS-ket at 3 mg/kg (N=8) or 50 mg/kg (N=6) versus saline (SAL, N=9). * p < 0.05 for 50 mg/kg RS-ket versus SAL for 100-120 min sample. Inset: Mean (+ SD) glutamate release (% baseline) indicating the significant main effect of 50 mg/kg RS-ket versus SAL; * p < 0.05. (B) Time course for S-ket at 1.5 mg/kg (N=9), 7.5 mg/kg (N=11) or 25 mg/kg (N=7) versus SAL (N=9), * p < 0.05 for 25 mg/kg RS-ket versus SAL for 0-20 min sample; **** p < 0.0005 for 25 mg/kg RS-ket versus SAL for 20-40 min sample. Inset: Mean (+ SD) glutamate release (% baseline) indicating the significant main effect of 25 mg/kg S-ket versus SAL and lower S-ket doses; * p < 0.05, ** p < 0.003, *** p < 0.001. (C) Positive control using K+-enriched artificial cerebrospinal fluid (aCSF) in a random sample of mice after completion of 2-hour microdialysate sampling for the main experiment (N = 5, mean + SD). Glutamate concentration was expressed as percent glutamate relative to the mean of the four samples collected before the K+-enriched aCSF.. ** p < 0.003 for glutamate release in the 40-60 min sample relative to baseline. (D) Drawings of coronal sections from the mouse brain atlas (Franklin and Paxinos, 2008) with filled circles depicting the locations of the microdialysis probes accepted as viable, as estimated by infusing methylene blue and brain sectioning.

## 4.4 Signalling proteins

For BDNF concentrations in dHIPP at 30 min post-ketamine, there was no effect of RS-ketamine ($p$ = 0.50, Fig. 4A). In contrast, there was an effect of S-ketamine ($F(2, 12) = 4.90$, $p < 0.03$, Fig. 4B): *post hoc* analysis indicated that 1.5 mg/kg induced an increase in dHIPP BDNF concentration relative to SAL ($p < 0.03$).

In all cases, the concentration-signal for phosphorylated mTOR was greater than for total mTOR (see Materials and Methods for explanation). Regarding RS-ketamine, for total mTOR concentration in dHIPP and mPFC at 30 min post-ketamine, there was no effectf: dHIPP $p$ = 0.30 (Fig. 5A) and mPFC $p$ = 0.94 (Fig. 5B), and this was also the case for phosphorylated mTOR specifically: dHIPP $p$ = 0.64 (Fig. 5A) and mPFC $p$ = 0.64 (Fig. 5B). Regarding S-ketamine, for total mTOR concentration in dHIPP and mPFC at 30 min post-ketamine, there was no effect: dHIPP $p$ = 0.51 (Fig. 5C) and mPFC $p$ = 0.67 (Fig. 5D), and no effect on phosphorylated mTOR: dHIPP $p$ = 0.39 (Fig. 5C) and mPFC $p$ = 0.57 (Fig. 5D).

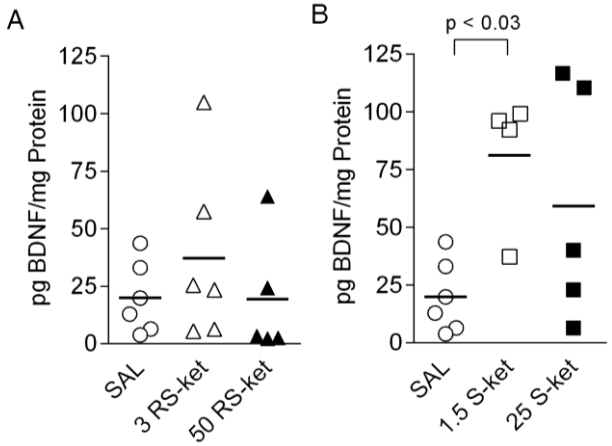


Figure 4. Scatter plots depicting effects of acute i.p. RS- or S-ketamine (-ket) on BDNF concentrations in the mouse dorsal hippocampus at 30 min post-injection. (A) RS-ket (3, 50 mg/kg) and (B) S-ket (1.5, 25 mg/kg) versus saline (SAL). Sample size was 4-6 per dose, with two 1.5 S-ket and one 25 S-ket samples nullified during sample processing.

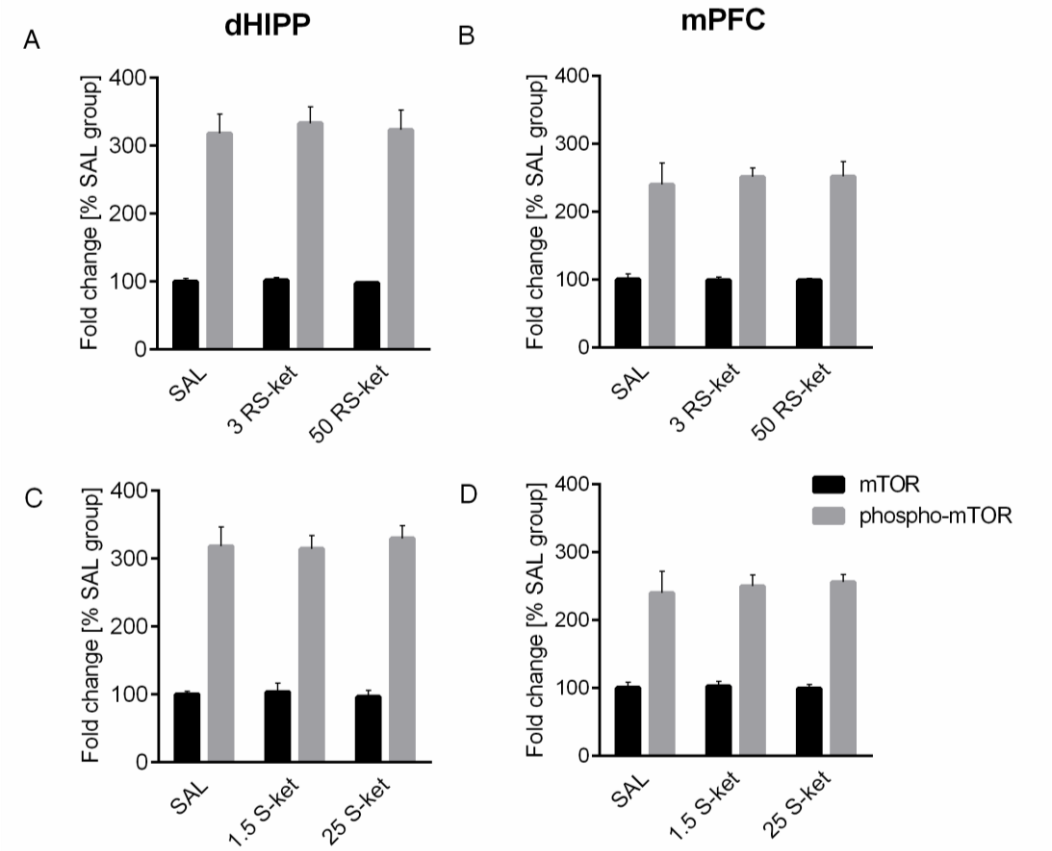



Figure 5. Effects of acute i.p. RS- or S-ketamine (-ket) on total- and phosphorylated-mTOR concentrations in the mouse brain at 30 min post-injection. (A-B) RS-ket in (A) dorsal hippocampus (dHIPP) and (B) medial prefrontal cortex (mPFC). (C-D) S-ket in (C) dHIPP and (D) mPFC. Sample size was 6 mice per dose, values are mean + SD.

# 5. DISCUSSION

This integrative study presents novel and important evidence that in otherwise non-manipulated C57BL/6 mice, the positive effect of acute ketamine in the mouse FST antidepressant screening test was specific to RS-ketamine and at a low-dose, and that this regimen was without effect on mPFC glutamate release or HIPP BDNF level - both of which were increased by other specific ketamine regimens - or on HIPP or mPFC mTOR phosphorylated or total level. This dissociation of behavioural, neurochemical and signalling protein effects challenges current hypotheses of ketamine mechanism-of-action as a pharmacotherapy for treatment-resistant depression.

With respect to the behavioural findings, the evidence that increased mobility (decreased floating) in the FST at 3 h post-treatment was specific to low-dose (3 mg/kg i.p.) RS-ketamine both confirms and expands on previous findings. For example, low-dose RS-ketamine increased mobility in the FST after intervals of 30 min, 3 h, 24 h and 1 week (Autry et al., 2011). The validity of the present FST as an antidepressant screening test was demonstrated with the tricyclic desipramine at 30-min post-treatment, as described previously (e.g. (Lucki et al., 2001)). For RS-ketamine the effect was borderline at 30 min and robust at 3 h. The equivalent low dose of the more potent S-enantiomer (1.5 mg/kg i.p.) had no effect on immobility at 3 h, as was the case with 7.5 mg/kg. These findings are consistent with the RS-ketamine effect not being mediated by NMDA-R antagonism and with off-target effects of the racemate, or R-ketamine specifically, being responsible. In a recent study, neonatal mice were exposed to the glucocorticoid receptor agonist dexamethasone, which increases FST immobility in juveniles, and the efficacies of R- or S-ketamine in reversing this effect were investigated. At 24 h post-injection with either enantiomer at 10 mg/kg i.p., both enantiomers decreased FST immobility to a similar extent; lower doses and shorter treatment-test intervals were not studied (Zhang et al., 2014). In the LH paradigm, low-dose RS-ketamine was without effect on 2-way escape behaviour at 3 h post-treatment, as were all other ketamine regimens investigated. The validity of this LH paradigm for antidepressant screening was demonstrated using the tricyclic imipramine at 30-min post-treatment. In the LH paradigm, repeated pre-exposure to inescapable electroshock leads to a gradual reduction in escape reactivity (motivational effort) across days; as such, the neurobiology underlying the escape deficit in the LH paradigm is unlikely to be equivalent to that underlying rapid-onset floating in the FST (Pryce et al., 2012). It has been previously reported that RS-ketamine at 2.5 mg/kg decreased escape failure (reversed LH) in a mouse LH paradigm, with ketamine administered immediately after the single inescapable electroshock session and 24-h prior to the escape test ((Maeng et al., 2008); personal communication). However, in light of the present findings, the possibility that ketamine inhibited the consolidation of LH rather than inducing its reversal,, should be investigated.

To our knowledge this is the first study of effects of low-dose ketamine on mPFC glutamate release in rodents. Both low-dose RS- and S-ketamine were without effect, whereas high-dose RS-ketamine increased mPFC glutamate release, as reported previously for rat (Lorrain et al., 2003; Moghaddam et al., 1997), and this was also the case for an equivalent high-dose of S-ketamine. The glutamate increase was more rapid and acute for S- than RS-ketamine, perhaps reflecting different pharmacokinetics in terms of transport to the brain. Therefore, the effect of RS-ketamine in the FST at

3 h post-treatment was not dependent on a detectable increase in extracellular glutamate release in mPFC during hours 1-2 post-treatment. This negative evidence for low-dose RS-ketamine is important given that hypotheses to-date on the mechanism-of-action of low-dose ketamine have had to extrapolate from findings obtained with medium-high ketamine doses. For example, increased mPFC glutamate release has been inferred to underlie the molecular and cellular actions of low-dose ketamine as an antidepressant, including increased glutamate-AMPA receptor activation, followed by increased BDNF release and mTOR signalling ((Duman and Li, 2012; Duman et al., 2012); see below). The present findings contradict this hypothesis, although despite the absence of increased extracellular glutamate it is still possible that there is a net increase in glutamate-AMPA activation relative to glutamate-NMDA activation, due to low-dose ketamine blocking of the latter glutamate receptor-type specifically.

It has been proposed that major down-stream effects of ketamine-induced increased glutamate-AMPA activation include increased expression and release of BDNF in the mPFC (e.g. (Duman and Li, 2012; Duman et al., 2012)). The intra-cellular BDNF level is viewed as a marker for general activity in signalling pathways promoting synaptic protein synthesis and synaptogenesis (Duman et al., 2012; Thoenen, 1995). Increased BDNF expression has been proposed as a relevant neurobiological effect of various antidepressants, including ketamine, and in this context the hippocampus has been a region of interest (Castren and Rantamaki, 2010). For technical reasons it was not possible to measure mPFC BDNF levels in the present study, but HIPP BDNF was measured. Low-dose RS-ketamine was without effect on the HIPP BDNF level at 30 min post-treatment, whereas low-dose S-ketamine increased it; high-dose RS- and S-ketamine were both without effect. This negative finding for low-dose RS-ketamine is in contrast to the HIPP BDNF increase in mice described by Autry et al. using the same dose and interval as those used here (Autry et al., 2011). In rat, HIPP BDNF was not increased by RS-ketamine at 5 mg/kg (low-dose) after 30 or 60 min and was increased by 10 mg/kg (medium-dose) (Garcia et al., 2008; Yang et al., 2013). Integrating the current and existing evidence, one interpretation is that, depending on other factors, central BDNF levels can be increased by ketamine and that S- is more potent than RS-ketamine in this respect. An additional major function assigned to BDNF, and in particular to its release, is activation of the BNDF-TrkB-Akt/ERK-mTOR pathway, leading to increased neuroplasticity (Duman et al., 2012; Li et al., 2010). mTOR is activated via phosphorylation, and in rat, RS-ketamine at 10 mg/kg increased the level of phosphorylated mTOR after 30 min in mPFC and HIPP (Li et al., 2010; Yang et al., 2013). In the present study there was no effect of any ketamine regimen on phosphorylated or total mTOR in mPFC or HIPP, including the 1.5 mg/kg S-ketamine dose that increased HIPP BDNF. It is important to note that there are technical differences between the present study and those that describe ketamine-induced mTOR phosphorylation which could contribute to study differences: these include homogenates (current study) versus synaptosomes (e.g. (Li et al., 2010)) and immunoassay (current study) versus western blot (e.g. (Autry et al., 2011)).

In summary, the present mouse study provides the first evidence that a low dose of acute RS-ketamine that functions as “antidepressant” in the FST screening test does so in the absence of increased mPFC glutamate release, increased HIPP BDNF or increased HIPP or mPFC phospho- or total-mTOR. The low dose of acute S-ketamine was without a behavioural or mPFC glutamate effect

despite increasing HIPP BDNF. The high doses of acute RS- and S-ketamine increased mPFC glutamate release, as predicted, but were without effect on behaviour, HIPP BDNF or HIPP and mPFC phospho- or total-mTOR. The current findings indicate that RS-ketamine affinity to receptors other than NMDA-R, which includes the μ-opiate, sigma and monoaminergic receptors (Kapur and Seeman, 2001; Robson et al., 2012; Wong et al., 1996), should be taken into account when interpreting its behavioural effects in the FST. Furthermore, these findings demonstrate the importance of studying enantiomer-specific effects of ketamine, both in animal models of depression and in human clinical studies, to elucidate the mechanism-of-action mediating between low-dose acute ketamine administration and remission from treatment-resistant depression.

# 6. ACKNOWLEDGEMENTS

We are extremely grateful to Patrizia Voehringer, Janosch Steinhauer, Renè Fuertig and Christoph Porazik for technical support with glutamate HPLC-FD, Myrtha Arnold and Susanne Wolfrum for technical support with mTOR immunoassay, and Franz Vollenweider and Milan Scheidegger for helpful expert discussions and comments on the manuscript. This research was funded by the Swiss National Science Foundation grant 31003A-141137. BF is an employee of Boehringer Ingelheim Pharma GmbH & Co. KG. CRP has received research funding from Servier International Research Institute and Boehringer Ingelheim Pharma GmbH & Co. KG, and the present work is unrelated to this grant.

# General discussion

## 1. INTEGRATION OF DIFFERENT NEURO-TRANSMITTER PATHWAYS UNDERLYING DEPRESSION

### 1.1 CYTOKINES

Among other factors such as obesity, early life stress, leaky gut and T cell dysfunction, psychosocial stress is likely to be the major source for an increased chronic inflammatory response (Haroon et al., 2012). The main focus has been centred on the sympathetic nervous system, which is activated during psychosocial stress and hypothesized to lead to an increase in peripheral inflammation. In humans, the pharmacological blockade of this system prevents the increased levels of cytokines in the blood (Mazzeo et al., 2001). The increased concentration of cytokines has been associated with depression in several studies (Dowlati et al., 2010) leading to the recent cytokine hypothesis of depression. In the present field of depression research, there is increasing evidence to support the hypothesis that the increased inflammatory activity is indeed an aetiological factor in depression, resulting in induction of various pathophysiological pathways that have been demonstrated to be associated with the disease.

Project A (see Chapter 2) has demonstrated that chronic social defeat (CSD) induced a peripheral stimulation of the pro-inflammatory response and a central cortico-limbic de-regulation in expression of genes involved in inflammatory and DA-ergic pathways; the latter specifically in the amygdala (see below section Cytokines and dopamine). These molecular changes are likely to be important contributors to the psychopathology-relevant behaviours exhibited by the stressed mice. In human it has been demonstrated that chronic psychosocial stress activates the peripheral immune system (Saveanu and Nemeroff, 2012), and in animals that it increases peripheral and central inflammatory responses (Kubera et al., 2011). There is human evidence that the peripheral activated inflammatory system leads to an increased inflammatory response in the brain; for example, peripheral administration of cytokine interferon-alpha (IFN-$\alpha$) increases interleukin 6 (IL-6) and monocyte chemoattractant protein-1 in the cerebrospinal fluid (Raison et al., 2009). A main on-going scientific discussion concerns the mechanism(s) underlying cytokine crossing of the blood-brain barrier. Several routes have been either demonstrated or proposed, including cytokines: (a) passing directly through leaks in the blood-brain barrier, (b) being actively transported by carrier proteins across the barrier, (c) activating perivascular macrophages and endothelial cells to release cytokines in the brain parenchyma, (d) recruiting immune cells which themselves cross the barrier, (e) stimulating nerve afferents in the periphery which stimulate inflammatory responses in specific brain regions (review by (Felger and Miller, 2012)) (Fig. 1, black frames). Further detailed studies will be required to establish which of these pathways is relevant in the case of mouse CSD.

In CSD mice, a specific number of de-regulated genes were found to be involved in inflammatory regulation processes. In the ventral hippocampus (vHIPP), 11 of the de-regulated genes were identified as having one or more of the pro-inflammatory cytokines TNF, IL-6 and IL-3 among their major regulators. In the amygdala (AMYG) and the medial prefrontal cortex (mPFC), two of the three top canonical pathways enriched by the de-regulated genes belonged to immuno-inflammation signalling processes. In depression, post mortem brain tissues show increased expression of genes belonging to pro-inflammatory and anti-inflammatory cytokine pathways in mPFC (Shelton et al., 2011). Additionally, a polymorphism in the TNF gene has also been correlated with depression (Bosker et al., 2011).

Further evidence that peripheral activation of cytokines leads to modifications in the brain is provided by human and animal studies demonstrating cytokine-induced psychopathology. For example, in cancer and hepatitis C patients treated with IFN-$\alpha$, symptoms of depression such as fatigue, cognitive impairment and psychomotor slowing are observed (Capuron and Miller, 2011). In primates and rodents, peripheral pro-inflammatory cytokine administration (e.g. IL-1β, TNF and INF-$\alpha$) induces depression-relevant behaviors such as social avoidance, decreased psychomotor activity and sleep alterations (Dantzer, 2001; Felger et al., 2007). In Project A, the CSD manipulation which led to increased blood levels of TNF and IL-6 also led to changes in behaviour, namely increased psychomotor fatigue, decreased psychomotor activity, increased fear of uncontrollable electroshock and increased helplessness to controllable electroshock. These are all depression-relevant behaviours, since fatigue and psychomotor retardation are classified as symptoms of depression (DSM-5, 2013; ICD-10, 1994), fear conditioning is potentiated in depressive patients (Nissen 2010), and generalised learned helplessness is a major theory for both onset and maintenance of depression (Abramson et al., 1978; Pryce et al., 2011).

The impact of immune-inflammation on monoamine neurotransmission, both on dopamine (DA) and serotonin (5-HT), has been discussed and demonstrated (Felger and Miller, 2012), and is supported by some of the major findings of this PhD thesis (see below sections Cytokines and dopamine, Cytokines and serotonin). Another central effect of pro-inflammatory processes, namely disruption of glutamate signalling via the NMDA receptor, will also be discussed (see section Cytokines and ketamine).

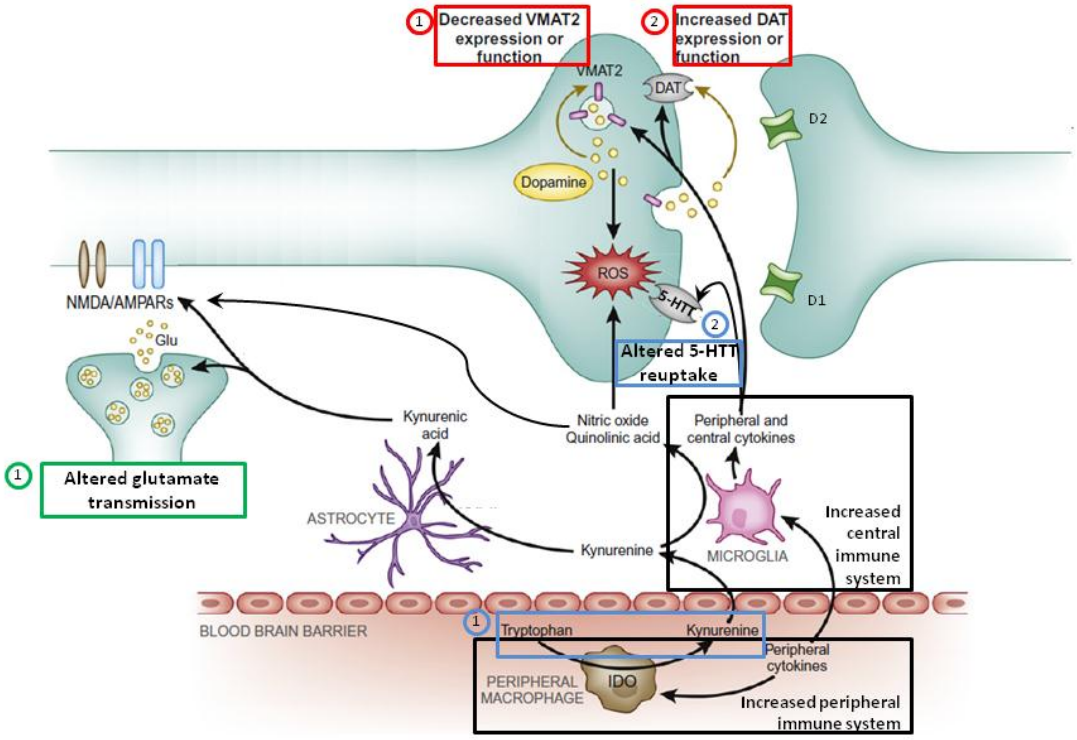



**Figure 1: Putative mechanisms via which cytokine-triggered inflammatory processes act on dopamine, serotonin and glutamate pathways**

Peripheral and central immune systems act through different routes on brain signalling pathways. Dopamine signalling (red frames): Central cytokines act on the expression of vesicular monoamines transporter 2 (VMAT2) and dopamine transporter (DAT). Serotonin signalling (blue frames): Peripheral cytokines shift tryptophan metabolism away from serotonin towards kynurenine (kynurenine pathway) and central cytokines interfere with the expression of the serotonin transporter (5-HTT). Glutamate signalling (green frames): The end products of the kynurenine pathways, kynurenic acid and quinolinic acid, modulate the NMDA receptor which results in modification of glutamatergic signalling.

5-HTT: serotonin transporter, AMPAR: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, D1/D2: dopamine receptor D1/D2, DAT: dopamine transporter, Glu: glutamate, IDO: indoleamine-pyrrole 2,3-dioxygenase, NMDAR: N-methyl-D-aspartate receptor, ROS: reactive oxygen species, VMAT2: vesicular monoamines transporter 2.

Figure adapted from (Felger and Miller, 2012).

## 1.2 CYTOKINES AND DOPAMINE

Concerning the hypothesis of DA deficiency in depression, there is growing evidence that psychosocial stress increases or decreases its availability depending on the type of stress (acute or chronic) and on the brain region (mPFC or nucleus accumbens (NAcc)). In rodents, single social defeat increased the DA turnover in the mPFC and to a larger extent than in the NAcc (Tanaka et al., 2012). This effect was attenuated in the mPFC by chronic exposure to the social stressor for 10 days. Chronic stress appears to reduce mesocortical DA activity, but to increase it in the mesoaccumbal pathway. Chronic social stress has been reported to either increase (Krishnan et al., 2007) or decrease (Tye et al., 2013) firing activity of DA neurons in the VTA, In the case where DA-ergic neuron activity was increased by CSD, elevated BDNF levels in the NAcc were also observed and were hypothesized to be responsible for the depression-relevant behaviours in these CSD mice (Krishnan et al., 2007). The NAcc receives a major input of BDNF from the VTA, and pharmacological blockade of BDNF in the NAcc prevents the CSD-induced behavioural effects (Krishnan et al., 2007). Additionally, BDNF administration into the NAcc increases depression-like behaviour after chronic social stress. A study of antidepressants in rat provides evidence in support of the mPFC and NAcc exhibiting opposite DA neuropharmacology: fluoxetine, imipramine and desipramine increase the extracellular DA concentration in the mPFC, but not in the NAcc (Tanda et al., 1994). In the study where chronic social stress decreased firing by VTA DA-ergic neurons, optogenetic activation of these neurons in chronically stressed mice reversed their depression-like behavioural phenotype, whereas optogenetic inhibition of these neurons induced such a phenotype in non-stressed mice. Furthermore, it was demonstrated that the DA-ergic neurons in the NAcc are essential for this regulation (Tye et al., 2013). *In vivo* data for DA levels are rare in depressive patients. However, in healthy humans, a positron emission tomography (PET) imaging study has shown increased DA release during stressful situations in the NAcc of young adult probands who reported having received relatively low levels of parental care during their childhood (Pruessner et al., 2004).

As discussed above, cytokines could be important mediators of the effects of stress on neurobiological changes and psychopathologies. Some rodent studies report altered concentration of DA after administration of IFN-α, with increases in cortical and hypothalamic regions (Kumai et al., 2000) and decreases in the AMYG (Kitagami et al., 2003). There are two major hypotheses of how cytokines can influence gene expression of DA-ergic signalling proteins, with both pertaining to reduced availability of DA: (a) reduction of the expression of vesicular monoamine transporter 2 (VAMT2) which packages DA in vesicles ready for release, and (b) increase in the expression of DA transporter (DAT, (Felger and Miller, 2012), see Fig. 1, red frames, Nr. 1 and 2). Another putative action of the inflammatory system is mediated by action on intracellular proteins such as through the oxidative reduction of the enzyme co-factor tetrahydrobiopterin (BH4), which is essential for DA synthesis. Activated microglia produce nitric oxide and quinolinic acid (QA, see section Cytokines and serotonin) which induce reactive oxygen species reducing the availability of BH4 and therefore also DA synthesis (Felger and Miller, 2012). In the AMGY of the CSD mice, a large number the de-regulated genes, specifically *Drd2*, *Adora2a*, *Gpr88*, *Darpp-32*, *Rgs9*, *Slc29a4 Gng7*, regulate or are regulated by DA. Interestingly, DA receptor D2 (DRD2) has been demonstrated to interact with

proteins encoded by a high proportion of the other genes de-regulated by CSD. Thus, it forms antagonistic dimers with adenosine A2 receptor (A2AR, (Boison et al., 2012)) and it is modulated by regulator of G Protein-9-2 (Rgs9-2) (Rahman et al., 2003). Dopamine- and cyclic AMP-regulated phosphoprotein-32 (Darpp-32) is a signalling phosphoprotein that is expressed mainly in DRD1 and DRD2 neurons (Bateup et al., 2010), and solute carrier family 29, member 4 (*Slc29a4*) encodes a protein that is expressed presynaptically and catalyses the uptake of DA and other monoamines (Dahlin et al., 2007). The expression of *Drd2* is high in central AMYG, as it is in the dorsal and ventral striata (Alheid and Heimer, 1988). In a post mortem study of depressed patients, altered density of DA-ergic receptors was also observed suggesting a dysfunction in the DA-ergic system; increased binding capacity of DRD2 and DRD3 and reduced DAT density were found in the AMYG (Klimek et al., 2002). Further evidence that dysfunction of DA plays an important role in depression is demonstrated by polymorphisms in DA-ergic genes that are associated with the disease (Antypa et al., 2013; Feng et al., 2010; Liou et al., 2009; Zou et al., 2012) or with specific symptoms such as fatigue (Lim et al., 2012; Malyuchenko et al., 2010). These include catechol-o-methyl transferase (*Comt*), *Dat1*, *Drd2*, GABA receptor subunit delta (*Gabrd)*, potassium channel, subfamily K, member 2 (*Kcnk2)* and 5-HT receptor 2a (*Htr2a*). Together with the findings in the CSD mice, there is growing evidence that proteins contributing to DA-ergic signalling, especially DRD2, contribute to the aetio-pathophysiology of depression, and constitute antidepressant targets with novel mechanism-of-action, (see Fig. 2, red frames, Nr. 1).

The AMYG is important in the formation and expression of the emotional responses acquired during fear conditioning, which were increased in the CSD mice. In rats, emotional stimuli release DA into the basolateral and central AMYG (Perez de la Mora et al., 2012), with the AMYG being innervated by DA-ergic terminals from both the VTA and substantia nigra (Fuxe et al., 2003). The NAcc and AMYG are central to the reward system and regulate motivational processes and the ability to experience pleasure (Dunlop and Nemeroff, 2007; Salamone and Correa, 2012). D-amphetamine, which increases DA synaptic release, raises the experience of reward to a greater extent in severely depressed patients relative to control subjects (Tremblay et al., 2002; Tremblay et al., 2005). In mice, increasing the DRD2 in the NAcc via viral vector techniques is sufficient to elevate motivation in several behavioural tests (Trifilieff et al., 2013). The CSD mice expressing less *Drd2* in the AMYG also show an increased fatigue indicating a motivational deficit. These results are in line with de-regulated DA-striatal functioning constituting a major underlying neuropathology (Capuron et al., 2007; Demyttenaere et al., 2005).

The CSD model provides evidence that psychosocial stress leads to an elevation of peripheral pro-inflammatory activity and to de-regulation of expression of immune-inflammation and DA-ergic genes in cortico-limbic brain regions that are important in the circuitry of depression-relevant behavioural states such as increased fear, helplessness and fatigue. Similar inflammatory response, molecular dysfunction and behavioural alteration can be observed in depressive patients, thereby providing evidence that the physiological states identified in the CSD model have high aetio-pathophysiological relevance for human depression.

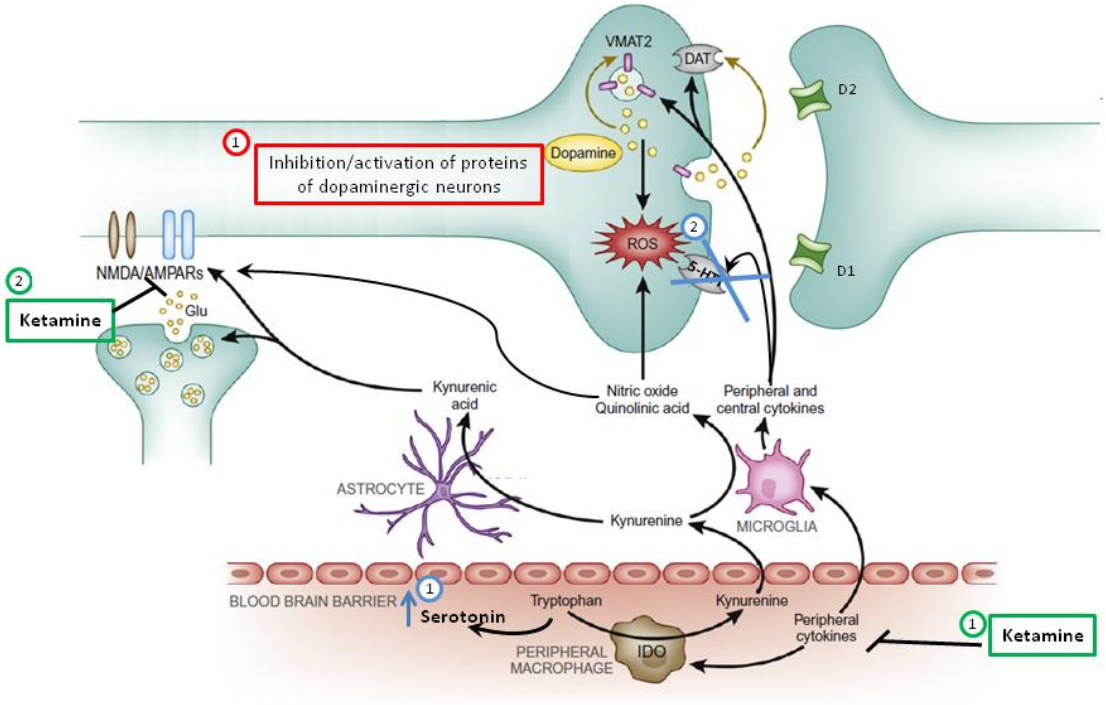



**Figure 2: Possible mechanisms-of-action for antidepressants acting on cytokine-triggered inflammatory processes**

Dopamine signalling (red frame): Elevated levels of cytokines that are induced via psychosocial stress modulate the expression of genes expressed specifically in dopaminergic neurons. Compounds activating or inhibiting the action of their corresponding proteins could be efficacious in treating depression. Serotonin signalling (blue pathways): Heterozygous serotonin transporter (5-HTT) knockout mice showed depression-relevant resilience after psychosocial stress. Increased serotonin in the blood could inhibit cytokine stimulation and action. Additionally, the action of cytokines on the 5-HTT protein in the brain is reduced due to the genotype which could mediate the anti-depressant effect. Glutamate signalling (green frames): The proposed anti-depressant effect of ketamine could be due to an inhibition of cytokine stimulation in the periphery or through the action on AMPA or non-NMDA receptors.

5-HTT: serotonin transporter, AMPAR: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, D1/D2: dopamine receptor D1/D2, DAT: dopamine transporter, Glu: glutamate, IDO: indoleamine-pyrrole 2,3-dioxygenase, NMDAR: N-methyl-D-aspartate receptor, ROS: reactive oxygen species, VMAT2: vesicular monoamines transporter 2.

Figure adapted from (Felger and Miller, 2012).

## 1.3 CYTOKINES AND SEROTONIN

There are several important lines of evidence that link the 5-HT deficiency and the pro-inflammatory cytokine hypotheses. It has been demonstrated that, in the periphery, increased pro-inflammatory cytokine levels lead to reduced 5-HT synthesis via the kynurenine pathway. The enzyme indoleamine-2,3-dioxygenase (IDO) is activated by cytokines such as TNF and INF-α (Dantzer, 2009) and is responsible (rate-limiting) for shifting tryptophan metabolism away from 5-HT towards kynurenine (KYN) and finally, to either kynurenic acid (KA) or QA (see Fig 1, blue frame, Nr. 1). Project B demonstrated suppression of CSD-induced TNF production in 5-HT transporter (5-HTT) heterozygous knockout (HET) mice. This suggests an association between the genotype and the peripheral inflammation response. Assuming that reduced 5-HTT leads to an overall increase in 5-HT, including in the periphery, the elevated 5-HT level could be responsible for a suppression of inflammatory response. This effect has been demonstrated *in vitro*: 5-HT decreased the levels of TNF and IL-6 in a human whole blood study (Kubera et al., 2005) (see Fig 2, blue arrow, Nr. 1). This suppressive effect could lead to the observed stress-resilience of HET mice of the 5-HTT KO strain relative to wildtype.

Cytokines and catabolites of the kynurenine pathway have effects on the 5-HT-ergic pathway in the brain. For example, IDO is present in macrophages and dendritic cells of the brain and its activity is increased during immune system activation (Wirleitner et al., 2003) e.g. lipopolysaccharide (LPS) activates IDO in the periphery and in the brain (Lestage et al., 2002). Furthermore, the metabolic end products KA and QA have opposing effects on the NMDA receptor (see section Cytokines and ketamine).

Interestingly, a direct effect of cytokines on the 5-HTT protein has been demonstrated *in vitro* and *in vivo* (Zhu et al., 2006; Zhu et al., 2010). IL-1β and TNF enhance 5-HTT activity i.e. 5-HT uptake in serotonergic cell lines. Mice deficient of 5-HTT or IL-1 receptor provide evidence that cytokines require 5-HTT expression to induce psychopathological behaviours (see Fig 1, blue frame, Nr. 2). According to these findings, humans, including those exposed to pro-inflammatory cytokine medication, who have high 5-HT activity exhibit lower pro-inflammatory IL-6 levels and lower depression scores (Raison 2009). Together with the absence of cytokine activation, the reduced expression of 5-HTT could play an important role in the reduced fear conditioning response observed in the 5-HTT HET relative to WT mice in Project B (see Fig 2, blue lines, Nr. 2).

There is a paradox in psychiatry between 5-HT and depression: on the one hand blocking 5-HTT is the basis of selective 5-HT reuptake inhibitor (SSRI) antidepressant, whilst on the other hand a 5-HTT gene polymorphism – namely the “short” (S) deletion insertion - that, similarly to SSRIs, reduces 5-HT reuptake, is currently the best-described genetic risk factor for environmental stress-induced depression. Meta-analysis yields strong statistical support for association between the S-allele x early-life stress and depression, suggesting a neurodevelopmental process. However, there is only marginal support for association between S x adult stress and depression, suggesting a net protective effect of the S allele against adult stress. Confounding results have been shown also with the 5-HTT KO mouse line. The majority of studies report an anxiolytic phenotype of 5-HTT HET and homozygous KO mice, but investigating gene x environmental (GxE) interactions various outcomes have been reported. Interestingly, stress during adolescence in 5-HTT HET mice has been reported to induce

anxious behaviour and depression-like responses in the probabilistic reversal learning task (Spinelli et al., 2013). Taken together, human and mice studies support the hypothesis that the S allele or partial deficiency of the 5-HTT possibly confer with adult stress resilience.

## 1.4 CYTOKINES AND KETAMINE

Recent studies in treatment-resistant depression patients have demonstrated that ketamine, a NMDA receptor antagonist, improves the symptoms (Berman et al., 2000; Zarate et al., 2006). The mechanism of anti-depressant action of ketamine is under investigation. Ketamine has been shown to be anti-inflammatory (Zunszain et al., 2013). In humans, ketamine administration inhibits the post-operative IL-6 inflammatory response (Dale et al., 2012) and suppresses experimentally induced TNF and IL-6 activation in the blood (Kawasaki 1999). In animals and *in vitro* studies, co-administration of ketamine with LPS suppresses TNF production (Lankveld et al., 2005; Taniguchi et al., 2003) (see Fig 2, green frame, Nr. 1). Ketamine could directly inhibit the expression of nuclear factor-kappa B which induces inflammation (Welters et al., 2010). However, as shown in rats, the doses of ketamine that were shown to suppress the cytokine levels are in the medium to high range (Taniguchi et al., 2003).

Another potential anti-inflammatory mechanism-of-action of ketamine involves the kynurenine pathway. As described above (see General introduction and section Cytokines and dopamine), cytokines induce a shift away from end product 5-HT towards QA and KA (see Fig 1, green frame, Nr. 1). These tryptophan-pathway catabolites act with opposing effects on the NMDA receptor: QA is an NMDA receptor agonist, whilst KA is an antagonist. QA also induces intracellular oxidative stress via activation of nitric oxide. In depressive patients, QA-KA disequilibrium is observed. The increase in QA and reduction in KA suggest a larger neurotoxic versus a lower neuroprotective effect of the kynurenine pathway during depression (Myint et al., 2007; Steiner et al., 2011).

Given that the CSD manipulation of Project A led to depression-relevant behaviours, this model could be applied to investigate ketamine. Specific doses (low, medium or high) could reverse the stress-induced peripheral cytokine response and reverse the psychopathological behaviours demonstrating its anti-depressant effect. Interestingly, the upstream analysis of the genes that were de-regulated in the mPFC of the CSD mice identified that both the NMDA receptor and ketamine are significant regulators. The ketamine-influenced genes, namely *Arc*, *Fos* and *Penk*, were all up-regulated in the CSD mice. Inhibition of the NMDA receptor leads to decreases in *Arc* mRNA (Link et al., 1995), and activation to increases of *Fos* mRNA (Herdegen and Leah, 1998). The findings suggest that ketamine could normalize the expression of these genes by balancing deregulation in the glutamate signalling of the mPFC which is responsible for some depression-relevant behaviours (see Fig 2, green frame, Nr. 2).

Project C demonstrated that a low-dose of either racemic (RS-) or S-ketamine did not change extracellular glutamate release in the mPFC. These results suggest that: (1) low-dose ketamine, even if it influences cortical NMDA:AMPA receptor signalling, does not do so by increasing the extracellular glutamate level; (2) the behavioural effects of low-dose RS-ketamine reported in this and in previous studies are independent of increased cortical glutamate release (Autry et al., 2011). Low-dose RS-ketamine did not modify the depression-relevant behaviour of learned helplessness (LH) in non-manipulated mice. However, it was positive in the antidepressant screening test of reduced immobility in the forced swim test (FST, for differences between FST and LH see the section Limitations of the thesis). The latter effect was not mimicked by the low-dose S-ketamine indicating a divergent effect from the RS-racemate suggesting the action on different receptors by the two enantiomers. This

divergence was additionally confirmed by an increase in hippocampal BDNF level induced only by low-dose S-ketamine.

Taken together, if ketamine has an anti-depressant effect this could be regulated via (a) non-NMDA receptors, (b) altered intracellular signalling, or (c) suppression of peripheral inflammatory response. Furthermore, these findings demonstrate the importance of studying enantiomer-specific effects of ketamine, both in animal models of depression and in human clinical studies, to elucidate the mechanism-of-action mediating between low-dose acute ketamine administration and remission from treatment-resistant depression.

# 2. LIMITATIONS OF THE THESIS

It is often discussed that psychosocial stress induced by CSD in mice is different and more severe than the psychosocial stress seen in humans (Nestler and Hyman, 2010) and additionally, that the ruminative aspects of aversive events in human are impossible to model in rodents. However, modifying the CSD protocol as done in Projects A and B removed all physical injuries and therefore reduced the severity of the psychosocial stress. This made the stress purely emotional and more aetiologically valid for translational research.

An anhedonic effect was not induced by CSD although several studies report this effect (Haque et al., 2012; Venzala et al., 2013). First, saccharin was used in the present study and not the normally used sucrose. This was done to avoid any nutritional effect that sucrose may have and to reduce the effect to a purely gustatory one. Second, the results show a high, almost 100% preference of the saccharin solution in all mice before the stress. It is not to exclude that by reducing the preference to around 80% with a different saccharin dose before the CSD session, as described in the literature with sucrose solution (Yu et al., 2011), an anhedonic effect in CSD mice could be observed. Third, since the CSD mice show an increased fatigue that suggests compromised effortful motivation, a test based on effort-dependent reward motivation would be expected to show differences between the groups. This would increase the construct validity of the model, since drugs acting on the DA-ergic system have been demonstrated to modify effortful choices in human (Wardle et al., 2011).

For the ketamine study in Project C, it has to be emphasized that the action of the NMDA receptor antagonist was tested in non-manipulated mice. In human studies, its anti-depressant effect was demonstrated in depressive patients. With respect to translational research, ketamine would need to be tested in terms of reversal of a prior-induced depression-relevant behaviour (see Outlook). However, the major aim of Project C was to address a number of open issues pertaining to the study of ketamine in non-manipulated mice.

The forced swim test is a widely-used screening test for antidepressants, but has marginal validity as a model for depression (Cryan and Mombereau, 2004). In the ketamine study, this test was used to replicate the results found in previous studies (e.g. (Autry et al., 2011)), and not for validating ketamine as an antidepressant. To provide more information about the effects of ketamine on the psychopathology of depression, the learned helplessness test was used.

Concerning neuroanatomy, the punching procedure of the regions of interest provided only a small amount of tissue; especially for the medial prefrontal cortex where only one biopsy per hemisphere was taken. For the analysis of both proteins (BDNF and mTOR), the lysed homogenate had to be split for the two kits which resulted in a loss of detectability of BDNF in this region.

Another general problem with the punching is the low spatial resolution. It is not possible to be more specific than in the range of 100ths of a millimetre. For example in the Next Generation Sequencing measurement in Project A, hierarchical clustering analysis demonstrated a region-specific sampling of biopsy (data not shown). But within the region, it would be impossible to separate different nuclei e.g. the central from the basolateral AMYG.

# 3. OUTLOOK

With respect to the long-term behavioural effects caused by CSD, it would be interesting to analyse the gene expression at the same time point, i.e. two weeks after the last CSD session. One would expect to have larger effects of gene expression alterations in the mPFC - the region that regulates the controllability which is compromised the most in the learned helplessness test at exactly this time point. In the AMYG, the de-regulation of the DA-ergic genes should persist and/or increase indicated by the sustained fatigue in the treadmill test. The results of the DA-ergic genes turned the focus on the reward system and on its centres such as dorsal and ventral striatum and ventral tegmental area. In future, further gene expression studies of CSD vs CON mice in these regions should be performed. Among others, the concentration of the neurotransmitters DA and 5-HT and their metabolites should be analysed in brain homogenates. Concerning the results of the reduced *Drd2* gene expression in the AMYG and the evidence that central and basolateral AMYG express non-overlapping populations of D1 and D2 receptor neurons (Perez de la Mora et al., 2012) it would be important to examine the structural changes of D1- and D2-expressing neurons in the AMYG with the help of immunohistochemichal techniques. Another possibility is that other regions with similar DA receptor distribution, such as the medium spiny neurons of the dorsal and ventral striatum, are also affected by CSD. A mouse magnetic resonance imaging and spectroscopy study should be performed. This would allow for the *in vivo* study of (1) the resting-state connectivity between cortical (mPFC), limbic regions (AMYG) and basal ganglia (dorsal and ventral striatum) and (2) depression-relevant neurotransmitters and their metabolites (e.g. GABA, glutamate). Further readout experiments could be conducted relating to specific symptoms of depression, including CSD effects on reward motivation measured with effort-dependent reward seeking and on architecture and homeostatic regulation of sleep. To provide more evidence on the immune-inflammatory state of CSD mice, activation of innate immune cells in the spleen and of microglia in specific brain regions should be measured using various approaches including fluorescent flow cytometry and immunohistochemistry. Additionally, the effects of specific cytokine administration (e.g. TNF) in the periphery or of viral over-expression in specific brain regions should be tested in terms of neurobiological and behavioural effects. Concerning the 5-HTT HET mice that underwent CSD, further brain regions (mPFC, AMYG and vHIPP) should be analysed for DA and 5-HT levels and turnover. This will allow for assessment of the extent of the effects of heterozygous knockout of the 5-HTT gene on DA and 5-HT. From the gene expression data in Project A, it is important to investigate whether the DA level of the CSD WT mice is altered relative to CON WT in the AMYG and, if so, whether this effect is modulated by knockout of one 5-HTT gene copy. Finally, the CSD model should be utilised to investigate the antidepressant effects and mechanism-of-action of different ketamine enantiomers, as well as other drug classes including anti-inflammatory low molecular weight compounds and biologics.

# References


Abramson, L.Y., Seligman, M.E., and Teasdale, J.D. (1978). Learned helplessness in humans: critique and reformulation. J Abnorm Psychol *87*, 49-74.

Alheid, G.F., and Heimer, L. (1988). New perspectives in basal forebrain organization of special relevance for neuropsychiatric disorders: the striatopallidal, amygdaloid, and corticopetal components of substantia innominata. Neuroscience *27*, 1-39.

Amat, J., Baratta, M.V., Paul, E., Bland, S.T., Watkins, L.R., and Maier, S.F. (2005). Medial prefrontal cortex determines how stressor controllability affects behavior and dorsal raphe nucleus. Nat Neurosci *8*, 365-371.

Anand, A., Li, Y., Wang, Y., Wu, J., Gao, S., Bukhari, L., Mathews, V.P., Kalnin, A., and Lowe, M.J. (2005). Activity and connectivity of brain mood regulating circuit in depression: a functional magnetic resonance study. Biol Psychiatry *57*, 1079-1088.

Anisman, H., and Merali, Z. (2001). Rodent models of depression: learned helplessness induced in mice. Curr Protoc Neurosci *Chapter 8*, Unit 8 10C.

Antypa, N., Calati, R., Souery, D., Pellegrini, S., Sentissi, O., Amital, D., Moser, U., Montgomery, S., Kasper, S., Zohar, J.*, et al.* (2013). Variation in the HTR1A and HTR2A genes and social adjustment in depressed patients. J Affect Disord *150*, 649-652.

Autry, A.E., Adachi, M., Nosyreva, E., Na, E.S., Los, M.F., Cheng, P.F., Kavalali, E.T., and Monteggia, L.M. (2011). NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. Nature *475*, 91-95.

Avgustinovich, D.F., Kovalenko, I.L., and Kudryavtseva, N.N. (2005). A model of anxious depression: persistence of behavioral pathology. Neurosci Behav Physiol *35*, 917-924.

Banasr, M., and Duman, R.S. (2008). Glial loss in the prefrontal cortex is sufficient to induce depressive-like behaviors. Biol Psychiatry *64*, 863-870.

Banks, W.A. (2006). The blood-brain barrier in psychoneuroimmunology. Neurol Clin *24*, 413-419.

Bartolomucci, A., Cabassi, A., Govoni, P., Ceresini, G., Cero, C., Berra, D., Dadomo, H., Franceschini, P., Dell'Omo, G., Parmigiani, S.*, et al.* (2009). Metabolic consequences and vulnerability to diet-induced obesity in male mice under chronic social stress. PLoS One *4*, e4331.

Bartolomucci, A., Carola, V., Pascucci, T., Puglisi-Allegra, S., Cabib, S., Lesch, K.P., Parmigiani, S., Palanza, P., and Gross, C. (2010). Increased vulnerability to psychosocial stress in heterozygous serotonin transporter knockout mice. Dis Model Mech *3*, 459-470.

Bartolomucci, A., Palanza, P., Sacerdote, P., Ceresini, G., Chirieleison, A., Panerai, A.E., and Parmigiani, S. (2003). Individual housing induces altered immuno-endocrine responses to psychological stress in male mice. Psychoneuroendocrinology *28*, 540-558.

Bartolomucci, A., Pederzani, T., Sacerdote, P., Panerai, A.E., Parmigiani, S., and Palanza, P. (2004). Behavioral and physiological characterization of male mice under chronic psychosocial stress. Psychoneuroendocrinology *29*, 899-910.

Bateup, H.S., Santini, E., Shen, W., Birnbaum, S., Valjent, E., Surmeier, D.J., Fisone, G., Nestler, E.J., and Greengard, P. (2010). Distinct subclasses of medium spiny neurons differentially regulate striatal motor behaviors. Proc Natl Acad Sci U S A *107*, 14845-14850.

Becker, J.A., Befort, K., Blad, C., Filliol, D., Ghate, A., Dembele, D., Thibault, C., Koch, M., Muller, J., Lardenois, A.*, et al.* (2008). Transcriptome analysis identifies genes with enriched expression in the mouse central extended amygdala. Neuroscience *156*, 950-965.

Bengel, D., Murphy, D.L., Andrews, A.M., Wichems, C.H., Feltner, D., Heils, A., Mossner, R., Westphal, H., and Lesch, K.P. (1998). Altered brain serotonin homeostasis and locomotor insensitivity to 3, 4-methylenedioxymethamphetamine ("Ecstasy") in serotonin transporter-deficient mice. Mol Pharmacol *53*, 649-655.

Berman, R.M., Cappiello, A., Anand, A., Oren, D.A., Heninger, G.R., Charney, D.S., and Krystal, J.H. (2000). Antidepressant effects of ketamine in depressed patients. Biol Psychiatry *47*, 351-354.

Berton, O., McClung, C.A., Dileone, R.J., Krishnan, V., Renthal, W., Russo, S.J., Graham, D., Tsankova, N.M., Bolanos, C.A., Rios, M.*, et al.* (2006). Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. Science *311*, 864-868.

Bierhaus, A., Wolf, J., Andrassy, M., Rohleder, N., Humpert, P.M., Petrov, D., Ferstl, R., von Eynatten, M., Wendt, T., Rudofsky, G.*, et al.* (2003). A mechanism converting psychosocial stress into mononuclear cell activation. Proc Natl Acad Sci U S A *100*, 1920-1925.

Blanchard, D.C., Griebel, G., and Blanchard, R.J. (2001). Mouse defensive behaviors: pharmacological and behavioral assays for anxiety and panic. Neurosci Biobehav Rev *25*, 205-218.

Boison, D., Singer, P., Shen, H.Y., Feldon, J., and Yee, B.K. (2012). Adenosine hypothesis of schizophrenia--opportunities for pharmacotherapy. Neuropharmacology *62*, 1527-1543.

Bosker, F.J., Hartman, C.A., Nolte, I.M., Prins, B.P., Terpstra, P., Posthuma, D., van Veen, T., Willemsen, G., DeRijk, R.H., de Geus, E.J.*, et al.* (2011). Poor replication of candidate genes for major depressive disorder using genome-wide association data. Mol Psychiatry *16*, 516-532.

Bradley, R.G., Binder, E.B., Epstein, M.P., Tang, Y., Nair, H.P., Liu, W., Gillespie, C.F., Berg, T., Evces, M., Newport, D.J.*, et al.* (2008). Influence of child abuse on adult depression: moderation by the corticotropin-releasing hormone receptor gene. Arch Gen Psychiatry *65*, 190-200.

Bradtmoller, M., Hartmann, C., Zietsch, J., Jaschke, S., Mautner, V.F., Kurtz, A., Park, S.J., Baier, M., Harder, A., Reuss, D.*, et al.* (2012). Impaired Pten expression in human malignant peripheral nerve sheath tumours. PLoS One *7*, e47595.

Brody, S.A., Geyer, M.A., and Large, C.H. (2003). Lamotrigine prevents ketamine but not amphetamine-induced deficits in prepulse inhibition in mice. Psychopharmacology (Berl) *169*, 240-246.

Brown, G.W., Ban, M., Craig, T.K., Harris, T.O., Herbert, J., and Uher, R. (2013). Serotonin transporter length polymorphism, childhood maltreatment, and chronic depression: a specific gene-environment interaction. Depress Anxiety *30*, 5-13.

Buck, K., Voehringer, P., and Ferger, B. (2009). Rapid analysis of GABA and glutamate in microdialysis samples using high performance liquid chromatography and tandem mass spectrometry. J Neurosci Methods *182*, 78-84.

Canli, T., Qiu, M., Omura, K., Congdon, E., Haas, B.W., Amin, Z., Herrmann, M.J., Constable, R.T., and Lesch, K.P. (2006). Neural correlates of epigenesis. Proc Natl Acad Sci U S A *103*, 16033-16038.

Capuron, L., and Miller, A.H. (2004). Cytokines and psychopathology: lessons from interferon-alpha. Biol Psychiatry *56*, 819-824.

Capuron, L., and Miller, A.H. (2011). Immune system to brain signaling: neuropsychopharmacological implications. Pharmacol Ther *130*, 226-238.

Capuron, L., Pagnoni, G., Demetrashvili, M.F., Lawson, D.H., Fornwalt, F.B., Woolwine, B., Berns, G.S., Nemeroff, C.B., and Miller, A.H. (2007). Basal ganglia hypermetabolism and symptoms of fatigue during interferon-alpha therapy. Neuropsychopharmacology *32*, 2384-2392.

Carlyle, B.C., Duque, A., Kitchen, R.R., Bordner, K.A., Coman, D., Doolittle, E., Papademetris, X., Hyder, F., Taylor, J.R., and Simen, A.A. (2012). Maternal separation with early weaning: a rodent model providing novel insights into neglect associated developmental deficits. Dev Psychopathol *24*, 1401-1416.

Carmichael, M.D., Davis, J.M., Murphy, E.A., Brown, A.S., Carson, J.A., Mayer, E.P., and Ghaffar, A. (2006). Role of brain IL-1beta on fatigue after exercise-induced muscle damage. Am J Physiol Regul Integr Comp Physiol *291*, R1344-1348.

Caspi, A., Hariri, A.R., Holmes, A., Uher, R., and Moffitt, T.E. (2010). Genetic sensitivity to the environment: the case of the serotonin transporter gene and its implications for studying complex diseases and traits. Am J Psychiatry *167*, 509-527.

Caspi, A., and Moffitt, T.E. (2006). Gene-environment interactions in psychiatry: joining forces with neuroscience. Nat Rev Neurosci *7*, 583-590.

Caspi, A., Sugden, K., Moffitt, T.E., Taylor, A., Craig, I.W., Harrington, H., McClay, J., Mill, J., Martin, J., Braithwaite, A.*, et al.* (2003). Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science *301*, 386-389.

Castren, E., and Rantamaki, T. (2010). The role of BDNF and its receptors in depression and antidepressant drug action: Reactivation of developmental plasticity. Dev Neurobiol *70*, 289-297.

Celver, J., Sharma, M., and Kovoor, A. (2012). D(2)-Dopamine receptors target regulator of G protein signaling 9-2 to detergent-resistant membrane fractions. J Neurochem *120*, 56-69.

Chiao, J.Y., and Blizinsky, K.D. (2010). Culture-gene coevolution of individualism-collectivism and the serotonin transporter gene. Proc Biol Sci *277*, 529-537.

Choudary, P.V., Molnar, M., Evans, S.J., Tomita, H., Li, J.Z., Vawter, M.P., Myers, R.M., Bunney, W.E., Jr., Akil, H., Watson, S.J.*, et al.* (2005). Altered cortical glutamatergic and GABAergic signal transmission with glial involvement in depression. Proc Natl Acad Sci U S A *102*, 15653-15658.

Cowen, P.J. (2008). Serotonin and depression: pathophysiological mechanism or marketing myth? Trends Pharmacol Sci *29*, 433-436.

Craig, A., Tran, Y., Wijesuriya, N., and Boord, P. (2006). A controlled investigation into the psychological determinants of fatigue. Biol Psychol *72*, 78-87.

Cruz, S.L., Soberanes-Chavez, P., Paez-Martinez, N., and Lopez-Rubalcava, C. (2009). Toluene has antidepressant-like actions in two animal models used for the screening of antidepressant drugs. Psychopharmacology (Berl) *204*, 279-286.

Cryan, J.F., Markou, A., and Lucki, I. (2002). Assessing antidepressant activity in rodents: recent developments and future needs. Trends Pharmacol Sci *23*, 238-245.

Cryan, J.F., and Mombereau, C. (2004). In search of a depressed mouse: utility of models for studying depression-related behavior in genetically modified mice. Mol Psychiatry *9*, 326-357.

D'Mello, C., Le, T., and Swain, M.G. (2009). Cerebral microglia recruit monocytes into the brain in response to tumor necrosis factoralpha signaling during peripheral organ inflammation. J Neurosci *29*, 2089-2102.

Dahlin, A., Xia, L., Kong, W., Hevner, R., and Wang, J. (2007). Expression and immunolocalization of the plasma membrane monoamine transporter in the brain. Neuroscience *146*, 1193-1211.

Dale, O., Somogyi, A.A., Li, Y., Sullivan, T., and Shavit, Y. (2012). Does intraoperative ketamine attenuate inflammatory reactivity following surgery? A systematic review and meta-analysis. Anesth Analg *115*, 934-943.

Dalton, E.J., and Heinrichs, R.W. (2005). Depression in multiple sclerosis: a quantitative review of the evidence. Neuropsychology *19*, 152-158.

Dantzer, R. (2001). Cytokine-induced sickness behavior: mechanisms and implications. Ann N Y Acad Sci *933*, 222-234.

Dantzer, R. (2009). Cytokine, sickness behavior, and depression. Immunol Allergy Clin North Am *29*, 247-264.

Dantzer, R., O'Connor, J.C., Freund, G.G., Johnson, R.W., and Kelley, K.W. (2008). From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci *9*, 46-56.

Dantzer, R., O'Connor, J.C., Lawson, M.A., and Kelley, K.W. (2011). Inflammation-associated depression: from serotonin to kynurenine. Psychoneuroendocrinology *36*, 426-436.

De Berardis, D., Conti, C.M., Serroni, N., Moschetta, F.S., Olivieri, L., Carano, A., Salerno, R.M., Cavuto, M., Farina, B., Alessandrini, M.*, et al.* (2010). The effect of newer serotonin-noradrenalin antidepressants on cytokine production: a review of the current literature. Int J Immunopathol Pharmacol *23*, 417-422.

de la Mora, M.P., Gallegos-Cari, A., Arizmendi-Garcia, Y., Marcellino, D., and Fuxe, K. (2010). Role of dopamine receptor mechanisms in the amygdaloid modulation of fear and anxiety: Structural and functional analysis. Prog Neurobiol *90*, 198-216.

De Luca, M.T., Meringolo, M., Spagnolo, P.A., and Badiani, A. (2012). The role of setting for ketamine abuse: clinical and preclinical evidence. Rev Neurosci *23*, 769-780.

Demyttenaere, K., De Fruyt, J., and Stahl, S.M. (2005). The many faces of fatigue in major depressive disorder. Int J Neuropsychopharmacol *8*, 93-105.

Disner, S.G., Beevers, C.G., Haigh, E.A., and Beck, A.T. (2011). Neural mechanisms of the cognitive model of depression. Nat Rev Neurosci *12*, 467-477.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics *29*, 15-21.

Dominguez-Lopez, S., Howell, R., and Gobbi, G. (2012). Characterization of serotonin neurotransmission in knockout mice: implications for major depression. Rev Neurosci *23*, 429-443.

Dowlati, Y., Herrmann, N., Swardfager, W., Liu, H., Sham, L., Reim, E.K., and Lanctot, K.L. (2010). A meta-analysis of cytokines in major depression. Biol Psychiatry *67*, 446-457.

Drevets, W.C. (2001). Neuroimaging and neuropathological studies of depression: implications for the cognitive-emotional features of mood disorders. Curr Opin Neurobiol *11*, 240-249.

DSM-5 (2013). Diagnostic and Statistical Manual of Mental Disorders, fifth ed. American Psychiatric Association, Washington, DC.

Duman, R.S., and Li, N. (2012). A neurotrophic hypothesis of depression: role of synaptogenesis in the actions of NMDA receptor antagonists. Philos Trans R Soc Lond B Biol Sci *367*, 2475-2484.

Duman, R.S., Li, N., Liu, R.J., Duric, V., and Aghajanian, G. (2012). Signaling pathways underlying the rapid antidepressant actions of ketamine. Neuropharmacology *62*, 35-41.

Dunlop, B.W., and Nemeroff, C.B. (2007). The role of dopamine in the pathophysiology of depression. Arch Gen Psychiatry *64*, 327-337.

Eisenberger, N.I., Inagaki, T.K., Rameson, L.T., Mashal, N.M., and Irwin, M.R. (2009). An fMRI study of cytokine-induced depressed mood and social pain: the role of sex differences. Neuroimage *47*, 881-890.

Elliott, R., Rubinsztein, J.S., Sahakian, B.J., and Dolan, R.J. (2002). The neural basis of mood-congruent processing biases in depression. Arch Gen Psychiatry *59*, 597-604.

Engel, K., Zhou, M., and Wang, J. (2004). Identification and characterization of a novel monoamine transporter in the human brain. J Biol Chem *279*, 50042-50049.

Fava, M., and Davidson, K.G. (1996). Definition and epidemiology of treatment-resistant depression. Psychiatr Clin North Am *19*, 179-200.

Felger, J.C., Alagbe, O., Hu, F., Mook, D., Freeman, A.A., Sanchez, M.M., Kalin, N.H., Ratti, E., Nemeroff, C.B., and Miller, A.H. (2007). Effects of interferon-alpha on rhesus monkeys: a nonhuman primate model of cytokine-induced depression. Biol Psychiatry *62*, 1324-1333.

Felger, J.C., and Miller, A.H. (2012). Cytokine effects on the basal ganglia and dopamine function: the subcortical source of inflammatory malaise. Front Neuroendocrinol *33*, 315-327.

Feng, Y., Kapornai, K., Kiss, E., Tamas, Z., Mayer, L., Baji, I., Daroczi, G., Benak, I., Kothencne, V.O., Dombovari, E.*, et al.* (2010). Association of the GABRD gene and childhood-onset mood disorders. Genes Brain Behav *9*, 668-672.

Foreyt, J.P., Brunner, R.L., Goodrick, G.K., Cutter, G., Brownell, K.D., and St Jeor, S.T. (1995). Psychological correlates of weight fluctuation. Int J Eat Disord *17*, 263-275.

Franklin, K.B.J., and Paxinos, G. (2008). The mouse brain in stereotaxic coordinates, 3rd edn (New York, Elseviers Inc.).

Fuxe, K., Jacobsen, K.X., Hoistad, M., Tinner, B., Jansson, A., Staines, W.A., and Agnati, L.F. (2003). The dopamine D1 receptor-rich main and paracapsular intercalated nerve cell groups of the rat amygdala: relationship to the dopamine innervation. Neuroscience *119*, 733-746.

Garcia, L.S., Comim, C.M., Valvassori, S.S., Reus, G.Z., Barbosa, L.M., Andreazza, A.C., Stertz, L., Fries, G.R., Gavioli, E.C., Kapczinski, F.*, et al.* (2008). Acute administration of ketamine induces antidepressant-like effects in the forced swimming test and increases BDNF levels in the rat hippocampus. Prog Neuropsychopharmacol Biol Psychiatry *32*, 140-144.

Golden, S.A., Covington, H.E., 3rd, Berton, O., and Russo, S.J. (2011). A standardized protocol for repeated social defeat stress in mice. Nat Protoc *6*, 1183-1191.

Gordon, R., Anantharam, V., Kanthasamy, A.G., and Kanthasamy, A. (2012). Proteolytic activation of proapoptotic kinase protein kinase Cdelta by tumor necrosis factor alpha death receptor signaling in dopaminergic neurons during neuroinflammation. J Neuroinflammation *9*, 82.

Gotlib, I.H., and Joormann, J. (2010). Cognition and depression: current status and future directions. Annu Rev Clin Psychol *6*, 285-312.

Gustafsson, K., Calounova, G., Hjelm, F., Kriz, V., Heyman, B., Gronvik, K.O., Mostoslavsky, G., and Welsh, M. (2011). Shb deficient mice display an augmented TH2 response in peripheral CD4+ T cells. BMC Immunol *12*, 3.

Haque, F.N., Lipina, T.V., Roder, J.C., and Wong, A.H. (2012). Social defeat interacts with Disc1 mutations in the mouse to affect behavior. Behav Brain Res *233*, 337-344.

Hariri, A.R., Mattay, V.S., Tessitore, A., Kolachana, B., Fera, F., Goldman, D., Egan, M.F., and Weinberger, D.R. (2002). Serotonin transporter genetic variation and the response of the human amygdala. Science *297*, 400-403.

Haroon, E., Raison, C.L., and Miller, A.H. (2012). Psychoneuroimmunology meets neuropsychopharmacology: translational implications of the impact of inflammation on behavior. Neuropsychopharmacology *37*, 137-162.

Harrison, N.A., Brydon, L., Walker, C., Gray, M.A., Steptoe, A., and Critchley, H.D. (2009). Inflammation causes mood changes through alterations in subgenual cingulate activity and mesolimbic connectivity. Biol Psychiatry *66*, 407-414.

Harrison, P.J. (2002). The neuropathology of primary mood disorder. Brain *125*, 1428-1449.

Heim, C., Shugart, M., Craighead, W.E., and Nemeroff, C.B. (2010). Neurobiological and psychiatric consequences of child abuse and neglect. Dev Psychobiol *52*, 671-690.

Heinrichs, S.C., Menzaghi, F., Merlo Pich, E., Britton, K.T., and Koob, G.F. (1995). The role of CRF in behavioral aspects of stress. Ann N Y Acad Sci *771*, 92-104.

Herdegen, T., and Leah, J.D. (1998). Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. Brain Res Brain Res Rev *28*, 370-490.

Hernandez, M.E., Mendieta, D., Perez-Tapia, M., Bojalil, R., Estrada-Garcia, I., Estrada-Parra, S., and Pavon, L. (2013). Effect of selective serotonin reuptake inhibitors and immunomodulator on cytokines levels: an alternative therapy for patients with major depressive disorder. Clin Dev Immunol *2013*, 267871.

Hirota, K., and Lambert, D.G. (1996). Ketamine: its mechanism(s) of action and unusual clinical uses. Br J Anaesth *77*, 441-444.

Holma, K.M., Melartin, T.K., Haukka, J., Holma, I.A., Sokero, T.P., and Isometsa, E.T. (2010). Incidence and predictors of suicide attempts in DSM-IV major depressive disorder: a five-year prospective study. Am J Psychiatry *167*, 801-808.

Holmes, A., Murphy, D.L., and Crawley, J.N. (2003). Abnormal behavioral phenotypes of serotonin transporter knockout mice: parallels with human anxiety and depression. Biol Psychiatry *54*, 953-959.

Hossain, J.L., Reinish, L.W., Kayumov, L., Bhuiya, P., and Shapiro, C.M. (2003). Underlying sleep pathology may cause chronic high fatigue in shift-workers. J Sleep Res *12*, 223-230.

Hustveit, O., Maurset, A., and Oye, I. (1995). Interaction of the chiral forms of ketamine with opioid, phencyclidine, sigma and muscarinic receptors. Pharmacol Toxicol *77*, 355-359.

Hyman, S.E. (2012). Revolution stalled. Sci Transl Med *4*, 155cm111.

ICD-10 (1994). nternational Statistical Classification of Diseases and Related Health Problems 10th Revision.

Ineichen, C., Sigrist, H., Spinelli, S., Lesch, K.P., Sautter, E., Seifritz, E., and Pryce, C.R. (2012). Establishing a probabilistic reversal learning test in mice: evidence for the processes mediating reward-stay and punishment-shift behaviour and for their modulation by serotonin. Neuropharmacology *63*, 1012-1021.

Insel, T.R., and Sahakian, B.J. (2012). Drug research: a plan for mental illness. Nature *483*, 269.

Ising, M., and Holsboer, F. (2007). CRH-sub-1 receptor antagonists for the treatment of depression and anxiety. Exp Clin Psychopharmacol *15*, 519-528.

Ising, M., Kunzel, H.E., Binder, E.B., Nickel, T., Modell, S., and Holsboer, F. (2005). The combined dexamethasone/CRH test as a potential surrogate marker in depression. Prog Neuropsychopharmacol Biol Psychiatry *29*, 1085-1093.

Jansen, F., Heiming, R.S., Lewejohann, L., Touma, C., Palme, R., Schmitt, A., Lesch, K.P., and Sachser, N. (2010). Modulation of behavioural profile and stress response by 5-HTT genotype and social experience in adulthood. Behav Brain Res *207*, 21-29.

Kapur, S., and Seeman, P. (2001). Ketamine has equal affinity for NMDA receptors and the high-affinity state of the dopamine D2 receptor. Biol Psychiatry *49*, 954-957.

Karg, K., Burmeister, M., Shedden, K., and Sen, S. (2011). The serotonin transporter promoter variant (5-HTTLPR), stress, and depression meta-analysis revisited: evidence of genetic moderation. Arch Gen Psychiatry *68*, 444-454.

Katalinic, N., Lai, R., Somogyi, A., Mitchell, P.B., Glue, P., and Loo, C.K. (2013). Ketamine as a new treatment for depression: a review of its efficacy and adverse effects. Aust N Z J Psychiatry *47*, 710-727.

Keller, M.C., Neale, M.C., and Kendler, K.S. (2007). Association of different adverse life events with distinct patterns of depressive symptoms. Am J Psychiatry *164*, 1521-1529; quiz 1622.

Kendler, K.S., Hettema, J.M., Butera, F., Gardner, C.O., and Prescott, C.A. (2003). Life event dimensions of loss, humiliation, entrapment, and danger in the prediction of onsets of major depression and generalized anxiety. Arch Gen Psychiatry *60*, 789-796.

Kendler, K.S., Karkowski, L.M., and Prescott, C.A. (1999). Causal relationship between stressful life events and the onset of major depression. Am J Psychiatry *156*, 837-841.

Kendler, K.S., Kuhn, J.W., Vittum, J., Prescott, C.A., and Riley, B. (2005). The interaction of stressful life events and a serotonin transporter polymorphism in the prediction of episodes of major depression: a replication. Arch Gen Psychiatry *62*, 529-535.

Kennedy, S.H., and Rizvi, S.J. (2009). Emerging drugs for major depressive disorder. Expert Opin Emerg Drugs *14*, 439-453.

Kiecolt-Glaser, J.K., Loving, T.J., Stowell, J.R., Malarkey, W.B., Lemeshow, S., Dickinson, S.L., and Glaser, R. (2005). Hostile marital interactions, proinflammatory cytokine production, and wound healing. Arch Gen Psychiatry *62*, 1377-1384.

Kitagami, T., Yamada, K., Miura, H., Hashimoto, R., Nabeshima, T., and Ohta, T. (2003). Mechanism of systemically injected interferon-alpha impeding monoamine biosynthesis in rats: role of nitric oxide as a signal crossing the blood-brain barrier. Brain Res *978*, 104-114.

Klimek, V., Schenck, J.E., Han, H., Stockmeier, C.A., and Ordway, G.A. (2002). Dopaminergic abnormalities in amygdaloid nuclei in major depression: a postmortem study. Biol Psychiatry *52*, 740-748.

Kling, M.A., Coleman, V.H., and Schulkin, J. (2009). Glucocorticoid inhibition in the treatment of depression: can we think outside the endocrine hypothalamus? Depress Anxiety *26*, 641-649.

Koolhaas, J.M., De Boer, S.F., De Rutter, A.J., Meerlo, P., and Sgoifo, A. (1997). Social stress in rats and mice. Acta Physiol Scand Suppl *640*, 69-72.

Krishnan, V., Han, M.H., Graham, D.L., Berton, O., Renthal, W., Russo, S.J., Laplant, Q., Graham, A., Lutter, M., Lagace, D.C., *et al.* (2007). Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. Cell *131*, 391-404.

Krishnan, V., and Nestler, E.J. (2008). The molecular neurobiology of depression. Nature *455*, 894-902.

Kubera, M., Kenis, G., Bosmans, E., Scharpe, S., and Maes, M. (2000). Effects of serotonin and serotonergic agonists and antagonists on the production of interferon-gamma and interleukin-10. Neuropsychopharmacology *23*, 89-98.

Kubera, M., Maes, M., Kenis, G., Kim, Y.K., and Lason, W. (2005). Effects of serotonin and serotonergic agonists and antagonists on the production of tumor necrosis factor alpha and interleukin-6. Psychiatry Res *134*, 251-258.

Kubera, M., Obuchowicz, E., Goehler, L., Brzeszcz, J., and Maes, M. (2011). In animal models, psychosocial stress-induced (neuro)inflammation, apoptosis and reduced neurogenesis are associated to the onset of depression. Prog Neuropsychopharmacol Biol Psychiatry *35*, 744-759.

Kucukibrahimoglu, E., Saygin, M.Z., Caliskan, M., Kaplan, O.K., Unsal, C., and Goren, M.Z. (2009). The change in plasma GABA, glutamine and glutamate levels in fluoxetine- or S-citalopram-treated female patients with major depression. Eur J Clin Pharmacol *65*, 571-577.

Kudoh, A., Takahira, Y., Katagai, H., and Takazawa, T. (2002). Small-dose ketamine improves the postoperative state of depressed patients. Anesth Analg *95*, 114-118, table of contents.

Kudryavtseva, N.N., Bakshtanovskaya, I.V., and Koryakina, L.A. (1991). Social model of depression in mice of C57BL/6J strain. Pharmacol Biochem Behav *38*, 315-320.

Kumai, T., Tateishi, T., Tanaka, M., Watanabe, M., Shimizu, H., and Kobayashi, S. (2000). Effect of interferon-alpha on tyrosine hydroxylase and catecholamine levels in the brain of rats. Life Sci *67*, 663-669.

Lankveld, D.P., Bull, S., Van Dijk, P., Fink-Gremmels, J., and Hellebrekers, L.J. (2005). Ketamine inhibits LPS-induced tumour necrosis factor-alpha and interleukin-6 in an equine macrophage cell line. Vet Res *36*, 257-262.

LeDoux, J.E. (2000). Emotion circuits in the brain. Annu Rev Neurosci *23*, 155-184.

Lesch, K.P., Bengel, D., Heils, A., Sabol, S.Z., Greenberg, B.D., Petri, S., Benjamin, J., Muller, C.R., Hamer, D.H., and Murphy, D.L. (1996). Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. Science *274*, 1527-1531.

Lesch, K.P., and Mossner, R. (1998). Genetically driven variation in serotonin uptake: is there a link to affective spectrum, neurodevelopmental, and neurodegenerative disorders? Biol Psychiatry *44*, 179-192.

Lestage, J., Verrier, D., Palin, K., and Dantzer, R. (2002). The enzyme indoleamine 2,3-dioxygenase is induced in the mouse brain in response to peripheral administration of lipopolysaccharide and superantigen. Brain Behav Immun *16*, 596-601.

Leventopoulos, M., Ruedi-Bettschen, D., Knuesel, I., Feldon, J., Pryce, C.R., and Opacka-Juffry, J. (2007). Long-term effects of early life deprivation on brain glia in Fischer rats. Brain Res *1142*, 119-126.

Leveque, J.C., Macias, W., Rajadhyaksha, A., Carlson, R.R., Barczak, A., Kang, S., Li, X.M., Coyle, J.T., Huganir, R.L., Heckers, S.*, et al.* (2000). Intracellular modulation of NMDA receptor function by antipsychotic drugs. J Neurosci *20*, 4011-4020.

Li, N., Lee, B., Liu, R.J., Banasr, M., Dwyer, J.M., Iwata, M., Li, X.Y., Aghajanian, G., and Duman, R.S. (2010). mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. Science *329*, 959-964.

Li, N., Liu, R.J., Dwyer, J.M., Banasr, M., Lee, B., Son, H., Li, X.Y., Aghajanian, G., and Duman, R.S. (2011). Glutamate N-methyl-D-aspartate receptor antagonists rapidly reverse behavioral and synaptic deficits caused by chronic stress exposure. Biol Psychiatry *69*, 754-761.

Li, S.C., Passow, S., Nietfeld, W., Schroder, J., Bertram, L., Heekeren, H.R., and Lindenberger, U. (2013). Dopamine modulates attentional control of auditory perception: DARPP-32 (PPP1R1B) genotype effects on behavior and cortical evoked potentials. Neuropsychologia *51*, 1649-1661.

Lim, J., Ebstein, R., Tse, C.Y., Monakhov, M., Lai, P.S., Dinges, D.F., and Kwok, K. (2012). Dopaminergic polymorphisms associated with time-on-task declines and fatigue in the Psychomotor Vigilance Test. PLoS One *7*, e33767.

Link, W., Konietzko, U., Kauselmann, G., Krug, M., Schwanke, B., Frey, U., and Kuhl, D. (1995). Somatodendritic expression of an immediate early gene is regulated by synaptic activity. Proc Natl Acad Sci U S A *92*, 5734-5738.

Liou, Y.J., Chen, T.J., Tsai, S.J., Yu, Y.W., Cheng, C.Y., and Hong, C.J. (2009). Support for the involvement of the KCNK2 gene in major depressive disorder and response to antidepressant treatment. Pharmacogenet Genomics *19*, 735-741.

Little, A. (2009). Treatment-resistant depression. Am Fam Physician *80*, 167-172.

Logue, S.F., Grauer, S.M., Paulsen, J., Graf, R., Taylor, N., Sung, M.A., Zhang, L., Hughes, Z., Pulito, V.L., Liu, F.*, et al.* (2009). The orphan GPCR, GPR88, modulates function of the striatal dopamine system: a possible therapeutic target for psychiatric disorders? Mol Cell Neurosci *42*, 438-447.

Lopez-Leon, S., Janssens, A.C., Gonzalez-Zuloeta Ladd, A.M., Del-Favero, J., Claes, S.J., Oostra, B.A., and van Duijn, C.M. (2008). Meta-analyses of genetic studies on major depressive disorder. Mol Psychiatry *13*, 772-785.

Lorrain, D.S., Baccei, C.S., Bristow, L.J., Anderson, J.J., and Varney, M.A. (2003). Effects of ketamine and N-methyl-D-aspartate on glutamate and dopamine release in the rat prefrontal cortex: modulation by a group II selective metabotropic glutamate receptor agonist LY379268. Neuroscience *117*, 697-706.

Lucki, I., Dalvi, A., and Mayorga, A.J. (2001). Sensitivity to the effects of pharmacologically selective antidepressants in different strains of mice. Psychopharmacology (Berl) *155*, 315-322.

Maeng, S., Zarate, C.A., Jr., Du, J., Schloesser, R.J., McCammon, J., Chen, G., and Manji, H.K. (2008). Cellular mechanisms underlying the antidepressant effects of ketamine: role of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors. Biol Psychiatry *63*, 349-352.

Maes, M. (1995). Evidence for an immune response in major depression: a review and hypothesis. Prog Neuropsychopharmacol Biol Psychiatry *19*, 11-38.

Maes, M. (2011). Depression is an inflammatory disease, but cell-mediated immune activation is the key component of depression. Prog Neuropsychopharmacol Biol Psychiatry *35*, 664-675.

Maes, M., Leonard, B.E., Myint, A.M., Kubera, M., and Verkerk, R. (2011). The new '5-HT' hypothesis of depression: cell-mediated immune activation induces indoleamine 2,3-dioxygenase, which leads to lower plasma tryptophan and an increased synthesis of detrimental tryptophan catabolites (TRYCATs), both of which contribute to the onset of depression. Prog Neuropsychopharmacol Biol Psychiatry *35*, 702-721.

Maes, M., Yirmyia, R., Noraberg, J., Brene, S., Hibbeln, J., Perini, G., Kubera, M., Bob, P., Lerer, B., and Maj, M. (2009). The inflammatory & neurodegenerative (I&ND) hypothesis of depression: leads for future research and new drug developments in depression. Metab Brain Dis *24*, 27-53.

Maier, S.F., and Seligman, M.E.P. (1976). Learned Helplessness: Theory and Evidence. Journal of Experimental Psychology: General *105*, 3-46.

Malyuchenko, N.V., Schegolkova, J.V., Kulikova, M.A., Timofeeva, M.A., Shlepzova, V.A., Sysoeva, O.V., Ivanitsky, A.M., and Tonevitsky, A.G. (2010). Effects of genetic variations in the dopaminergic system on fatigue in humans: gender aspects. Bull Exp Biol Med *149*, 226-232.

Maren, S., Phan, K.L., and Liberzon, I. (2013). The contextual brain: implications for fear conditioning, extinction and psychopathology. Nat Rev Neurosci *14*, 417-428.

Marques-Vidal, P., Bochud, M., Bastardot, F., Luscher, T., Ferrero, F., Gaspoz, J.M., Paccaud, F., Urwyler, A., von Kanel, R., Hock, C.*, et al.* (2011). Levels and determinants of inflammatory biomarkers in a Swiss population-based sample (CoLaus study). PLoS One *6*, e21002.

Mathews, T.A., Fedele, D.E., Coppelli, F.M., Avila, A.M., Murphy, D.L., and Andrews, A.M. (2004). Gene dose-dependent alterations in extraneuronal serotonin but not dopamine in mice with reduced serotonin transporter expression. J Neurosci Methods *140*, 169-181.

Mazzeo, R.S., Donovan, D., Fleshner, M., Butterfield, G.E., Zamudio, S., Wolfel, E.E., and Moore, L.G. (2001). Interleukin-6 response to exercise and high-altitude exposure: influence of alpha-adrenergic blockade. J Appl Physiol (1985) *91*, 2143-2149.

McDade, T.W., Hawkley, L.C., and Cacioppo, J.T. (2006). Psychosocial and behavioral predictors of inflammation in middle-aged and older adults: the Chicago health, aging, and social relations study. Psychosom Med *68*, 376-381.

Merali, Z., Du, L., Hrdina, P., Palkovits, M., Faludi, G., Poulter, M.O., and Anisman, H. (2004). Dysregulation in the suicide brain: mRNA expression of corticotropin-releasing hormone receptors and GABA(A) receptor subunits in frontal cortical brain region. J Neurosci *24*, 1478-1485.

Miller, A.H., Maletic, V., and Raison, C.L. (2009). Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. Biol Psychiatry *65*, 732-741.

Mion, G., and Villevieille, T. (2013). Ketamine pharmacology: an update (pharmacodynamics and molecular aspects, recent findings). CNS Neurosci Ther *19*, 370-380.

Mitterschiffthaler, M.T., Williams, S.C., Walsh, N.D., Cleare, A.J., Donaldson, C., Scott, J., and Fu, C.H. (2008). Neural basis of the emotional Stroop interference effect in major depression. Psychol Med *38*, 247-256.

Moghaddam, B., Adams, B., Verma, A., and Daly, D. (1997). Activation of glutamatergic neurotransmission by ketamine: a novel step in the pathway from NMDA receptor blockade to dopaminergic and cognitive disruptions associated with the prefrontal cortex. J Neurosci *17*, 2921-2927.

Monleon, S., D'Aquila, P., Parra, A., Simon, V.M., Brain, P.F., and Willner, P. (1995). Attenuation of sucrose consumption in mice by chronic mild stress and its restoration by imipramine. Psychopharmacology (Berl) *117*, 453-457.

Mulinari, S. (2012). Monoamine theories of depression: historical impact on biomedical research. J Hist Neurosci *21*, 366-392.

Mullen, P.E., Martin, J.L., Anderson, J.C., Romans, S.E., and Herbison, G.P. (1996). The long-term impact of the physical, emotional, and sexual abuse of children: a community study. Child Abuse Negl *20*, 7-21.

Murphy, D.L., and Lesch, K.P. (2008). Targeting the murine serotonin transporter: insights into human neurobiology. Nat Rev Neurosci *9*, 85-96.

Murphy, F.C., Michael, A., Robbins, T.W., and Sahakian, B.J. (2003). Neuropsychological impairment in patients with major depressive disorder: the effects of feedback on task performance. Psychol Med *33*, 455-467.

Myint, A.M., Kim, Y.K., Verkerk, R., Scharpe, S., Steinbusch, H., and Leonard, B. (2007). Kynurenine pathway in major depression: evidence of impaired neuroprotection. J Affect Disord *98*, 143-151.

Narayanan, V., Heiming, R.S., Jansen, F., Lesting, J., Sachser, N., Pape, H.C., and Seidenbecher, T. (2011). Social defeat: impact on fear extinction and amygdala-prefrontal cortical theta synchrony in 5-HTT deficient mice. PLoS One *6*, e22600.

Narita, M., Yoshizawa, K., Aoki, K., Takagi, M., Miyatake, M., and Suzuki, T. (2001). A putative sigma1 receptor antagonist NE-100 attenuates the discriminative stimulus effects of ketamine in rats. Addict Biol *6*, 373-376.

Neill, J.C., Barnes, S., Cook, S., Grayson, B., Idris, N.F., McLean, S.L., Snigdha, S., Rajagopal, L., and Harte, M.K. (2010). Animal models of cognitive dysfunction and negative symptoms of schizophrenia: focus on NMDA receptor antagonism. Pharmacol Ther *128*, 419-432.

Nestler, E.J., and Hyman, S.E. (2010). Animal models of neuropsychiatric disorders. Nat Neurosci *13*, 1161-1169.

Nissen, C., Holz, J., Blechert, J., Feige, B., Riemann, D., Voderholzer, U., and Normann, C. (2010). Learning as a model for neural plasticity in major depression. Biol Psychiatry *68*, 544-552.

Nouwen, A., Winkley, K., Twisk, J., Lloyd, C.E., Peyrot, M., Ismail, K., and Pouwer, F. (2010). Type 2 diabetes mellitus as a risk factor for the onset of depression: a systematic review and meta-analysis. Diabetologia *53*, 2480-2486.

O'Hara, R., and Hallmayer, J.F. (2007). Serotonin transporter polymorphism and stress: a view across the lifespan. Curr Psychiatry Rep *9*, 173-175.

Oeckl, P., and Ferger, B. (2012). Simultaneous LC-MS/MS analysis of the biomarkers cAMP and cGMP in plasma, CSF and brain tissue. J Neurosci Methods *203*, 338-343.

Ong, J.C., Brody, S.A., Large, C.H., and Geyer, M.A. (2005). An investigation of the efficacy of mood stabilizers in rodent models of prepulse inhibition. J Pharmacol Exp Ther *315*, 1163-1171.

Owens, M., Goodyer, I.M., Wilkinson, P., Bhardwaj, A., Abbott, R., Croudace, T., Dunn, V., Jones, P.B., Walsh, N.D., Ban, M.*, et al.* (2012). 5-HTTLPR and early childhood adversities moderate cognitive and emotional processing in adolescence. PLoS One *7*, e48482.

Pace, T.W., Mletzko, T.C., Alagbe, O., Musselman, D.L., Nemeroff, C.B., Miller, A.H., and Heim, C.M. (2006). Increased stress-induced inflammatory responses in male patients with major depression and increased early life stress. Am J Psychiatry *163*, 1630-1633.

Palme, R., Touma, C., Arias, N., Dominchin, M., and Lepschy, M. (2013). Steroid extraction: Get the best out of faecal samples. Wiener Tierärztl Mschrift - Vet Med Austria *100*, 238-246.

Parker, K.J., Schatzberg, A.F., and Lyons, D.M. (2003). Neuroendocrine aspects of hypercortisolism in major depression. Horm Behav *43*, 60-66.

Paul, R., Schaaff, N., Padberg, F., Moller, H.J., and Frodl, T. (2009). Comparison of racemic ketamine and S-ketamine in treatment-resistant major depression: report of two cases. World J Biol Psychiatry *10*, 241-244.

Perez-Tejada, J., Arregi, A., Gomez-Lazaro, E., Vegas, O., Azpiroz, A., and Garmendia, L. (2013). Coping with chronic social stress in mice: hypothalamic-pituitary-adrenal/ sympathetic-adrenal-medullary axis activity, behavioral changes and effects of antalarmin treatment: implications for the study of stress-related psychopathologies. Neuroendocrinology *98*, 73-88.

Perez de la Mora, M., Gallegos-Cari, A., Crespo-Ramirez, M., Marcellino, D., Hansson, A.C., and Fuxe, K. (2012). Distribution of dopamine D(2)-like receptors in the rat amygdala and their role in the modulation of unconditioned fear and anxiety. Neuroscience *201*, 252-266.

Pizzagalli, D.A., Holmes, A.J., Dillon, D.G., Goetz, E.L., Birk, J.L., Bogdan, R., Dougherty, D.D., Iosifescu, D.V., Rauch, S.L., and Fava, M. (2009). Reduced caudate and nucleus accumbens response to rewards in unmedicated individuals with major depressive disorder. Am J Psychiatry *166*, 702-710.

Porsolt, R.D., Le Pichon, M., and Jalfre, M. (1977). Depression: a new animal model sensitive to antidepressant treatments. Nature *266*, 730-732.

Price, J.L., and Drevets, W.C. (2010). Neurocircuitry of mood disorders. Neuropsychopharmacology *35*, 192-216.

Pruessner, J.C., Champagne, F., Meaney, M.J., and Dagher, A. (2004). Dopamine release in response to a psychological stress in humans and its relationship to early life maternal care: a positron emission tomography study using [11C]raclopride. J Neurosci *24*, 2825-2831.

Pryce, C.R., Azzinnari, D., Sigrist, H., Gschwind, T., Lesch, K.P., and Seifritz, E. (2012). Establishing a learned-helplessness effect paradigm in C57BL/6 mice: behavioural evidence for emotional, motivational and cognitive effects of aversive uncontrollability per se. Neuropharmacology *62*, 358-372.

Pryce, C.R., Azzinnari, D., Spinelli, S., Seifritz, E., Tegethoff, M., and Meinlschmidt, G. (2011). Helplessness: a systematic translational review of theory and evidence for its relevance to understanding and treating depression. Pharmacol Ther *132*, 242-267.

Pryce, C.R., Bettschen, D., Bahr, N.I., and Feldon, J. (2001). Comparison of the effects of infant handling, isolation, and nonhandling on acoustic startle, prepulse inhibition, locomotion, and HPA activity in the adult rat. Behav Neurosci *115*, 71-83.

Pryce, C.R., Dettling, A.C., Spengler, M., Schnell, C.R., and Feldon, J. (2004). Deprivation of parenting disrupts development of homeostatic and reward systems in marmoset monkey offspring. Biol Psychiatry *56*, 72-79.

Pryce, C.R., and Feldon, J. (2003). Long-term neurobehavioural impact of the postnatal environment in rats: manipulations, effects and mediating mechanisms. Neurosci Biobehav Rev *27*, 57-71.

Pryce, C.R., and Klaus, F. (2013). Translating the evidence for gene association with depression into mouse models of depression-relevant behaviour: current limitations and future potential. Neurosci Biobehav Rev *37*, 1380-1402.

Pryce, C.R., and Seifritz, E. (2011). A translational research framework for enhanced validity of mouse models of psychopathological states in depression. Psychoneuroendocrinology *36*, 308-329.

Quibell, R., Prommer, E.E., Mihalyo, M., Twycross, R., and Wilcock, A. (2011). Ketamine*. J Pain Symptom Manage *41*, 640-649.

Quintana, A., Sanz, E., Wang, W., Storey, G.P., Guler, A.D., Wanat, M.J., Roller, B.A., La Torre, A., Amieux, P.S., McKnight, G.S.*, et al.* (2012). Lack of GPR88 enhances medium spiny neuron activity and alters motor- and cue-dependent behaviors. Nat Neurosci *15*, 1547-1555.

Rahman, Z., Schwarz, J., Gold, S.J., Zachariou, V., Wein, M.N., Choi, K.H., Kovoor, A., Chen, C.K., DiLeone, R.J., Schwarz, S.C.*, et al.* (2003). RGS9 modulates dopamine signaling in the basal ganglia. Neuron *38*, 941-952.

Raison, C.L., Borisov, A.S., Majer, M., Drake, D.F., Pagnoni, G., Woolwine, B.J., Vogt, G.J., Massung, B., and Miller, A.H. (2009). Activation of central nervous system inflammatory pathways by interferon-alpha: relationship to monoamines and depression. Biol Psychiatry *65*, 296-303.

Raison, C.L., Dantzer, R., Kelley, K.W., Lawson, M.A., Woolwine, B.J., Vogt, G., Spivey, J.R., Saito, K., and Miller, A.H. (2010). CSF concentrations of brain tryptophan and kynurenines during immune stimulation with IFN-alpha: relationship to CNS immune responses and depression. Mol Psychiatry *15*, 393-403.

Raison, C.L., Rutherford, R.E., Woolwine, B.J., Shuo, C., Schettler, P., Drake, D.F., Haroon, E., and Miller, A.H. (2013). A randomized controlled trial of the tumor necrosis factor antagonist infliximab for treatment-resistant depression: the role of baseline inflammatory biomarkers. JAMA Psychiatry *70*, 31-41.

Rajkowska, G., and Miguel-Hidalgo, J.J. (2007). Gliogenesis and glial pathology in depression. CNS Neurol Disord Drug Targets *6*, 219-233.

Reis, F.L., Masson, S., de Oliveira, A.R., and Brandao, M.L. (2004). Dopaminergic mechanisms in the conditioned and unconditioned fear as assessed by the two-way avoidance and light switch-off tests. Pharmacol Biochem Behav *79*, 359-365.

Reus, G.Z., Stringari, R.B., Ribeiro, K.F., Ferraro, A.K., Vitto, M.F., Cesconetto, P., Souza, C.T., and Quevedo, J. (2011). Ketamine plus imipramine treatment induces antidepressant-like behavior and increases CREB and BDNF protein levels and PKA and PKC phosphorylation in rat brain. Behav Brain Res *221*, 166-171.

Rhen, T., and Cidlowski, J.A. (2005). Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. N Engl J Med *353*, 1711-1723.

Richards, D. (2011). Prevalence and clinical course of depression: a review. Clin Psychol Rev *31*, 1117-1125.

Richelson, E. (1996). Synaptic effects of antidepressants. J Clin Psychopharmacol *16*, 1S-7S; discussion 7S-9S.

Robson, M.J., Elliott, M., Seminerio, M.J., and Matsumoto, R.R. (2012). Evaluation of sigma (sigma) receptors in the antidepressant-like effects of ketamine in vitro and in vivo. Eur Neuropsychopharmacol *22*, 308-317.

Rodgers, R.J., and Cole, J.C. (1993). Anxiety enhancement in the murine elevated plus maze by immediate prior exposure to social stressors. Physiol Behav *53*, 383-388.

Ruhe, H.G., Mason, N.S., and Schene, A.H. (2007). Mood is indirectly related to serotonin, norepinephrine and dopamine levels in humans: a meta-analysis of monoamine depletion studies. Mol Psychiatry *12*, 331-359.

Russo, S.J., and Nestler, E.J. (2013). The brain reward circuitry in mood disorders. Nat Rev Neurosci *14*, 609-625.

Salamone, J.D., and Correa, M. (2012). The mysterious motivational functions of mesolimbic dopamine. Neuron *76*, 470-485.

Sanacora, G., Zarate, C.A., Krystal, J.H., and Manji, H.K. (2008). Targeting the glutamatergic system to develop novel, improved therapeutics for mood disorders. Nat Rev Drug Discov *7*, 426-437.

Sandi, C., Merino, J.J., Cordero, M.I., Touyarot, K., and Venero, C. (2001). Effects of chronic stress on contextual fear conditioning and the hippocampal expression of the neural cell adhesion molecule, its polysialylation, and L1. Neuroscience *102*, 329-339.

Sasaki, K., Yamasaki, T., Omotuyi, I.O., Mishina, M., and Ueda, H. (2013). Age-dependent dystonia in striatal Ggamma7 deficient mice is reversed by the dopamine D2 receptor agonist pramipexole. J Neurochem *124*, 844-854.

Saveanu, R.V., and Nemeroff, C.B. (2012). Etiology of depression: genetic and environmental factors. Psychiatr Clin North Am *35*, 51-71.

Savignac, H.M., Finger, B.C., Pizzo, R.C., O'Leary, O.F., Dinan, T.G., and Cryan, J.F. (2011). Increased sensitivity to the effects of chronic social defeat stress in an innately anxious mouse strain. Neuroscience *192*, 524-536.

Savitz, J., Frank, M.B., Victor, T., Bebak, M., Marino, J.H., Bellgowan, P.S., McKinney, B.A., Bodurka, J., Kent Teague, T., and Drevets, W.C. (2013). Inflammation and neurological disease-related genes are differentially expressed in depressed patients with mood disorders and correlate with morphometric and functional imaging abnormalities. Brain Behav Immun *31*, 161-171.

Schlaepfer, T.E., Cohen, M.X., Frick, C., Kosel, M., Brodesser, D., Axmacher, N., Joe, A.Y., Kreft, M., Lenartz, D., and Sturm, V. (2008). Deep brain stimulation to reward circuitry alleviates anhedonia in refractory major depression. Neuropsychopharmacology *33*, 368-377.

Schmidt, M.V., Scharf, S.H., Liebl, C., Harbich, D., Mayer, B., Holsboer, F., and Muller, M.B. (2010). A novel chronic social stress paradigm in female mice. Horm Behav *57*, 415-420.

Serdar, K.L., Mazzeo, S.E., Mitchell, K.S., Aggen, S.H., Kendler, K.S., and Bulik, C.M. (2011). Correlates of weight instability across the lifespan in a population-based sample. Int J Eat Disord *44*, 506-514.

Sharp, T., and Cowen, P.J. (2011). 5-HT and depression: is the glass half-full? Curr Opin Pharmacol *11*, 45-51.

Shelton, R.C., Claiborne, J., Sidoryk-Wegrzynowicz, M., Reddy, R., Aschner, M., Lewis, D.A., and Mirnics, K. (2011). Altered expression of genes involved in inflammation and apoptosis in frontal cortex in major depression. Mol Psychiatry *16*, 751-762.

Siegle, G.J., Steinhauer, S.R., Thase, M.E., Stenger, V.A., and Carter, C.S. (2002). Can't shake that feeling: event-related fMRI assessment of sustained amygdala activity in response to emotional information in depressed individuals. Biol Psychiatry *51*, 693-707.

Singer, P., Wei, C.J., Chen, J.F., Boison, D., and Yee, B.K. (2013). Deletion of striatal adenosine A(2A) receptor spares latent inhibition and prepulse inhibition but impairs active avoidance learning. Behav Brain Res *242*, 54-61.

Spinelli, S., Müller, T., Friedel, M., Sigrist, H., Lesch, K.P., Henkelman, M., Rudin, M., Seifritz, E., and Pryce, C.R. (2013). Effects of repeated adolescent stress and serotonin transporter gene partial knockout in mice on behaviors and brain structures relevant to major depression. Front Behav Neurosci *7*, 1-15.

Steiner, J., Walter, M., Gos, T., Guillemin, G.J., Bernstein, H.G., Sarnyai, Z., Mawrin, C., Brisch, R., Bielau, H., Meyer zu Schwabedissen, L.*, et al.* (2011). Severe depression is associated with increased microglial quinolinic acid in subregions of the anterior cingulate gyrus: evidence for an immune-modulated glutamatergic neurotransmission? J Neuroinflammation *8*, 94.

Strigo, I.A., Simmons, A.N., Matthews, S.C., Craig, A.D., and Paulus, M.P. (2008). Association of major depressive disorder with altered functional brain response during anticipation and processing of heat pain. Arch Gen Psychiatry *65*, 1275-1284.

Sullivan, P.F., and Consortium (2013). A mega-analysis of genome-wide association studies for major depressive disorder. Mol Psychiatry *18*, 497-511.

Sullivan, P.F., Neale, M.C., and Kendler, K.S. (2000). Genetic epidemiology of major depression: review and meta-analysis. Am J Psychiatry *157*, 1552-1562.

Szanto, K., Mulsant, B.H., Houck, P., Dew, M.A., and Reynolds, C.F., 3rd (2003). Occurrence and course of suicidality during short-term treatment of late-life depression. Arch Gen Psychiatry *60*, 610-617.

Tanaka, K., Furuyashiki, T., Kitaoka, S., Senzai, Y., Imoto, Y., Segi-Nishida, E., Deguchi, Y., Breyer, R.M., Breyer, M.D., and Narumiya, S. (2012). Prostaglandin E2-mediated attenuation of mesocortical dopaminergic pathway is critical for susceptibility to repeated social defeat stress in mice. J Neurosci *32*, 4319-4329.

Tanda, G., Carboni, E., Frau, R., and Di Chiara, G. (1994). Increase of extracellular dopamine in the prefrontal cortex: a trait of drugs with antidepressant potential? Psychopharmacology (Berl) *115*, 285-288.

Taniguchi, T., Takemoto, Y., Kanakura, H., Kidani, Y., and Yamamoto, K. (2003). The dose-related effects of ketamine on mortality and cytokine responses to endotoxin-induced shock in rats. Anesth Analg *97*, 1769-1772.

Thoenen, H. (1995). Neurotrophins and neuronal plasticity. Science *270*, 593-598.

Touma, C., Sachser, N., Mostl, E., and Palme, R. (2003). Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. Gen Comp Endocrinol *130*, 267-278.

Trapnell, C., Hendrickson, D.G., Sauvageau, M., Goff, L., Rinn, J.L., and Pachter, L. (2013). Differential analysis of gene regulation at transcript resolution with RNA-seq. Nat Biotechnol *31*, 46-53.

Tremblay, L.K., Naranjo, C.A., Cardenas, L., Herrmann, N., and Busto, U.E. (2002). Probing brain reward system function in major depressive disorder: altered response to dextroamphetamine. Arch Gen Psychiatry *59*, 409-416.

Tremblay, L.K., Naranjo, C.A., Graham, S.J., Herrmann, N., Mayberg, H.S., Hevenor, S., and Busto, U.E. (2005). Functional neuroanatomical substrates of altered reward processing in major depressive disorder revealed by a dopaminergic probe. Arch Gen Psychiatry *62*, 1228-1236.

Trifilieff, P., Feng, B., Urizar, E., Winiger, V., Ward, R.D., Taylor, K.M., Martinez, D., Moore, H., Balsam, P.D., Simpson, E.H.*, et al.* (2013). Increasing dopamine D2 receptor expression in the adult nucleus accumbens enhances motivation. Mol Psychiatry *18*, 1025-1033.

Trullas, R., and Skolnick, P. (1990). Functional antagonists at the NMDA receptor complex exhibit antidepressant actions. Eur J Pharmacol *185*, 1-10.

Tye, K.M., Mirzabekov, J.J., Warden, M.R., Ferenczi, E.A., Tsai, H.C., Finkelstein, J., Kim, S.Y., Adhikari, A., Thompson, K.R., Andalman, A.S.*, et al.* (2013). Dopamine neurons modulate neural encoding and expression of depression-related behaviour. Nature *493*, 537-541.

Tyring, S., Gottlieb, A., Papp, K., Gordon, K., Leonardi, C., Wang, A., Lalla, D., Woolley, M., Jahreis, A., Zitnik, R.*, et al.* (2006). Etanercept and clinical outcomes, fatigue, and depression in psoriasis: double-blind placebo-controlled randomised phase III trial. Lancet *367*, 29-35.

Venzala, E., Garcia-Garcia, A.L., Elizalde, N., and Tordera, R.M. (2013). Social vs. environmental stress models of depression from a behavioural and neurochemical approach. Eur Neuropsychopharmacol *23*, 697-708.

Vignali, D.A. (2000). Multiplexed particle-based flow cytometric assays. J Immunol Methods *243*, 243-255.

Voehringer, P., Fuertig, R., and Ferger, B. (2013). A novel liquid chromatography/tandem mass spectrometry method for the quantification of glycine as biomarker in brain microdialysis and cerebrospinal fluid samples within 5min. J Chromatogr B Analyt Technol Biomed Life Sci *939*, 92-97.

Vollenweider, F.X., and Kometer, M. (2010). The neurobiology of psychedelic drugs: implications for the treatment of mood disorders. Nat Rev Neurosci *11*, 642-651.

Vollenweider, F.X., Leenders, K.L., Oye, I., Hell, D., and Angst, J. (1997). Differential psychopathology and patterns of cerebral glucose utilisation produced by (S)- and (R)-ketamine in healthy volunteers using positron emission tomography (PET). Eur Neuropsychopharmacol *7*, 25-38.

Walter, M., Henning, A., Grimm, S., Schulte, R.F., Beck, J., Dydak, U., Schnepf, B., Boeker, H., Boesiger, P., and Northoff, G. (2009). The relationship between aberrant neuronal activation in the pregenual anterior cingulate, altered glutamatergic metabolism, and anhedonia in major depression. Arch Gen Psychiatry *66*, 478-486.

Wardle, M.C., Treadway, M.T., Mayo, L.M., Zald, D.H., and de Wit, H. (2011). Amping up effort: effects of d-amphetamine on human effort-based decision-making. J Neurosci *31*, 16597-16602.

Warner-Schmidt, J.L., Vanover, K.E., Chen, E.Y., Marshall, J.J., and Greengard, P. (2011). Antidepressant effects of selective serotonin reuptake inhibitors (SSRIs) are attenuated by antiinflammatory drugs in mice and humans. Proc Natl Acad Sci U S A *108*, 9262-9267.

Weinstein, A.A., Deuster, P.A., Francis, J.L., Beadling, C., and Kop, W.J. (2010). The role of depression in short-term mood and fatigue responses to acute exercise. Int J Behav Med *17*, 51-57.

Weiss, J.M., and et al. (1981). Behavioral depression produced by an uncontrollable stressor: Relationship to norepinephrine, dopamine, and serotonin levels in various regions of rat brain. Brain Research Reviews *3*, 167-205.

Welters, I.D., Hafer, G., Menzebach, A., Muhling, J., Neuhauser, C., Browning, P., and Goumon, Y. (2010). Ketamine inhibits transcription factors activator protein 1 and nuclear factor-kappaB, interleukin-8 production, as well as CD11b and CD16 expression: studies in human leukocytes and leukocytic cell lines. Anesth Analg *110*, 934-941.

Willner, P. (1997). Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. Psychopharmacology (Berl) *134*, 319-329.

Willner, P. (2005). Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. Neuropsychobiology *52*, 90-110.

Wirleitner, B., Rudzite, V., Neurauter, G., Murr, C., Kalnins, U., Erglis, A., Trusinskis, K., and Fuchs, D. (2003). Immune activation and degradation of tryptophan in coronary heart disease. Eur J Clin Invest *33*, 550-554.

Wittchen, H.U., Jacobi, F., Rehm, J., Gustavsson, A., Svensson, M., Jonsson, B., Olesen, J., Allgulander, C., Alonso, J., Faravelli, C*., et al.* (2011). The size and burden of mental disorders and other disorders of the brain in Europe 2010. Eur Neuropsychopharmacol *21*, 655-679.

Wong, C.S., Cherng, C.H., Luk, H.N., Ho, S.T., and Tung, C.S. (1996). Effects of NMDA receptor antagonists on inhibition of morphine tolerance in rats: binding at mu-opioid receptors. Eur J Pharmacol *297*, 27-33.

Yang, C., Hu, Y.M., Zhou, Z.Q., Zhang, G.F., and Yang, J.J. (2013). Acute administration of ketamine in rats increases hippocampal BDNF and mTOR levels during forced swimming test. Ups J Med Sci *118*, 3-8.

Yu, T., Guo, M., Garza, J., Rendon, S., Sun, X.L., Zhang, W., and Lu, X.Y. (2011). Cognitive and neural correlates of depression-like behaviour in socially defeated mice: an animal model of depression with cognitive dysfunction. Int J Neuropsychopharmacol *14*, 303-317.

Zarate, C.A., Jr., Singh, J.B., Carlson, P.J., Brutsche, N.E., Ameli, R., Luckenbaugh, D.A., Charney, D.S., and Manji, H.K. (2006). A randomized trial of an N-methyl-D-aspartate antagonist in treatment-resistant major depression. Arch Gen Psychiatry *63*, 856-864.

Zhang, J.C., Li, S.X., and Hashimoto, K. (2014). R (-)-ketamine shows greater potency and longer lasting antidepressant effects than S (+)-ketamine. Pharmacol Biochem Behav *116*, 137-141.

Zhu, C.B., Blakely, R.D., and Hewlett, W.A. (2006). The proinflammatory cytokines interleukin-1beta and tumor necrosis factor-alpha activate serotonin transporters. Neuropsychopharmacology *31*, 2121-2131.

Zhu, C.B., Lindler, K.M., Owens, A.W., Daws, L.C., Blakely, R.D., and Hewlett, W.A. (2010). Interleukin-1 receptor activation by systemic lipopolysaccharide induces behavioral despair linked to MAPK regulation of CNS serotonin transporters. Neuropsychopharmacology *35*, 2510-2520.

Zimmermann, P., Bruckl, T., Nocon, A., Pfister, H., Binder, E.B., Uhr, M., Lieb, R., Moffitt, T.E., Caspi, A., Holsboer, F*., et al.* (2011). Interaction of FKBP5 gene variants and adverse life events in predicting depression onset: results from a 10-year prospective community study. Am J Psychiatry *168*, 1107-1116.

Zobel, A.W., Nickel, T., Kunzel, H.E., Ackl, N., Sonntag, A., Ising, M., and Holsboer, F. (2000). Effects of the high-affinity corticotropin-releasing hormone receptor 1 antagonist R121919 in major depression: the first 20 patients treated. J Psychiatr Res *34*, 171-181.

Zou, Y.F., Wang, F., Feng, X.L., Li, W.F., Tian, Y.H., Tao, J.H., Pan, F.M., and Huang, F. (2012). Association of DRD2 gene polymorphisms with mood disorders: a meta-analysis. J Affect Disord *136*, 229-237.

Zunszain, P.A., Horowitz, M.A., Cattaneo, A., Lupi, M.M., and Pariante, C.M. (2013). Ketamine: synaptogenesis, immunomodulation and glycogen synthase kinase-3 as underlying mechanisms of its antidepressant properties. Mol Psychiatry *18*, 1236-1241.

# Acknowledgements

In almost four years, I had the opportunity to meet excellent scientists, to work with interesting people and to make new friends. It was an exciting time from which I will take with me many joyful memories for the future. Thanks to all of you.

In particular, I would like to start thanking my supervisor PD. Dr. Christopher Pryce, for his scientific expertise and guidance, his everlasting disposition to help, his patience and nonetheless for his British humour. Special thank goes to Prof. Erich Seifritz for his support and scientific inputs on the manuscripts. I would like to thank Prof. Hanns Möhler and Prof. Markus Rudin who accepted to co-examine this thesis and were very helpful in discussing my project at the committee meetings and Prof. Markus Fendt for reading and reviewing this thesis.

Without the industrial partner Boehringer Ingelheim Pharma GmbH & Co. KG. (Biberach, Germany), the diversity of the results of this thesis would not have been possible. For the Next Generation Sequencing data I would like to thank Tobias Hildebrandt, German Leparc and Bastian Hengerer and for the HPLC-FC measurements in the microdialysis Boris Ferger. I appreciated very much the help that was provided to me by René Fürtig and Christoph Porazik; and the technical support and the good time by Janosch Steinhauer. Thanks to Rupert Palme from the University of Veterinary Medicine in Vienna who was always willing to analyze our animal samples.

Many thanks to all master students for their appreciated contribution to this project, namely Tilo Gschwind, Simon Stähli and Kilian Jörg.

Thanks to all former and present members of the PLaTRAD group. In particular, thanks to Hannes Sigrist for his entertaining skills that never compromised his extraordinary professionalism. I want to thank Federica Klaus, Flurin Cathomas and Christian Ineichen that besides the valuable scientific inputs could also give me support when I needed it the most. Thanks to Giorgio Bergamini and Simona Spinelli for bringing a homey Italian atmosphere to the lab.

To all the animal caretakers of the animal facility at the August Forel-Strasse goes a special thank, in particular to Ramiro Valero and Björn Henz. It is a hard work downstairs and without them my research would not have been possible. Many thanks to the whole neurology laboratory group of Prof. Weller who solved many of my problems even at short notice and let me experience many enjoyable

moments. Even if it is not part of my final project, I appreciated very much the help and expertise of Jean-Charles Paterna and Severin Schwendener on the plasmid construction.

This project was financed by the Swiss National Fond (project number: 31003A-130499 and 31003A-141137), and an additional funding for the attendance at the 11th World Congress of Biological Psychiatry in Kyoto (Japan) was provided by the International PhD Program of the Neuroscience Centre Zurich.

Many thanks to my sister Marisa and her whole family who provided an immense motivational support that was very important to me. For many happy and comfortable moments and for a constant help I would like to thank Lea, Roman and the whole bunch of people belonging to the “Herde”. The final and most important thank goes to my parents, Rosa and Demetrio. I devote this work to them: for having supported me financially during my studies, for having backed me up in every decision I’ve made, and especially, for having let me become the person who I am today.

# Curriculum vitae


## AZZINNARI
## Damiano

Born: 2nd of January 1982
Citizen of Niederlenz (AG)


## Education

**University of Zurich, Switzerland**
PhD project — 5/2010 - 4/2014
Preclinical Laboratory for Translational Research into Affective Disorders, Zurich University Hospital for Psychiatry

**Swiss Federal Institute of Technology Zurich, Switzerland**
Teaching certificate — 4/2005 - 10/2008

**Swiss Federal Institute of Technology Zurich, Switzerland**
Master in neuroscience — 4/2005 - 4/2007
*Title of thesis: In vitro* characterization of mouse tissue plasminogen activator-dependent brain-derived neurotrophic factor processing
Laboratory of Behavioural Neurobiology

**Imperial College London, United Kingdom**
Erasmus Exchange semester with focus in neurobiology — 10/2004 - 3/2005

**Swiss Federal Institute of Technology Zurich, Switzerland**
Bachelor in biology — 10/2002 - 9/2004

**Alte Kantonsschule Aarau, Switzerland**
Mathematics- & science-oriented high school (Typus C) — 8/1998 - 7/2002

# Appendix 1

Pharmacology & Therapeutics 132 (2011) 242–267

Contents lists available at SciVerse ScienceDirect

Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/pharmthera

Associate editor: S.L. Andersen

# Helplessness: A systematic translational review of theory and evidence for its relevance to understanding and treating depression

Christopher R. Pryce [a,*], Damiano Azzinnari [a], Simona Spinelli [a], Erich Seifritz [a], Marion Tegethoff [b], Gunther Meinlschmidt [b,c]

[a] Clinic for Affective Disorders, University Clinic of Psychiatry, Zurich, Switzerland
[b] Division of Clinical Psychology and Psychotherapy, Department of Psychology, University of Basel, Switzerland
[c] Division of Epidemiology and Health Psychology, Department of Psychology, University of Basel, Switzerland

ARTICLE INFO

*Keywords:*
Depression
Helplessness
Learned helplessness
Rat
Mouse
Human
Anti-depressant

ABSTRACT

Helplessness is a major concept in depression and a major theme in preclinical and clinical depression research. For example, in rodents and humans, the learned helplessness (LH) effect describes a specific deficit in behaviour to control aversive stimuli that is induced by prior exposure to uncontrollable aversive stimuli. The LH effect is objective and valid in that the cause of the behavioural deficit, namely uncontrollability, is clear; furthermore, the deficit induced is underlain by emotional, motivational and cognitive processes that are relevant to depression psychopathology. As a further example, helplessness, hopelessness, external locus of control and causal attribution are inter-related and major themes in psychological theories (primarily cognitive theories) of depression. Despite this broad interest in helplessness, it can be argued that its potential usefulness as a scientific and clinical concept has so far not been investigated optimally, including with respect to its application in research aimed at development of improved anti-depressant pharmacotherapy. The first aim of this review was to describe and integrate the psychological evidence and the neurobiological evidence for the LH effect in rodents and healthy humans and for helplessness in depressed patients. The second aim was to conduct three systematic reviews, namely of rodent studies of the LH effect, rodent studies of effects of psychopharmacological agents on the LH effect, and human studies of efficacy of pharmacotherapeutic and psychotherapeutic treatment on helplessness in depressed patients. With respect to the first aim, the major findings are: the specificity of the LH effect in otherwise non-manipulated rodents and healthy humans has been under-estimated, and the LH effect is a specific learned aversive uncontrollability (LAU) effect. There is theoretical and empirical support for a model in which a specific LAU effect induced by a life event of major emotional significance can function as an aetiological factor for generalised helplessness which can in turn function as an aetiological and maintenance factor for depression. However, to date such models have focused on cognitive mediating processes whereas it is emotional–motivational–cognitive processes (as proposed for the LAU effect) that need to be invoked and understood. The evidence is for analogous neural processes underlying the LAU effect in rodents and healthy humans and helplessness in depression, with the ventro-medial prefrontal cortex exhibiting aversive uncontrollability-dependent activity. With respect to the second aim, the major findings are: the LAU effect is demonstrated quite consistently using a number of different paradigms in rat but is poorly studied in mouse. The rat LAU effect can be reversed by chronic administration of monoamine reuptake inhibitors. The effects of antidepressants on human helplessness have been scarcely studied to-date. The major conclusion is that the LAU effect and generalised helplessness constitute major neuropsychological concepts of high value to future translational research aimed at increased understanding of depression and development of novel, improved antidepressant treatments.



**Contents**



* Corresponding author at: Preclinical Laboratory for Translational Research into Affective Disorders, Clinic for Affective Disorders and General Psychiatry, Psychiatric University Hospital Zurich, August Forel-Strasse 7, CH-8008 Zurich, Switzerland. Tel.: +41 44 634 8921.
*E-mail address:* Christopher.pryce@bli.uzh.ch (C.R. Pryce).

0163-7258/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.pharmthera.2011.06.006

## 1. Introduction

### 1.1. Background

Mood disorders, especially unipolar depressive disorders, such as major depressive disorder (MDD) and dysthymia, are among the most prevalent mental disorders (Kessler, Berglund, et al., 2005; Kessler, Chiu, et al., 2005; Kessler, Demler, et al., 2005). They place substantial burden on individuals with these disorders and on society (Lopez et al., 2006), and their prevalence is still increasing (Mathers & Loncar, 2006). Moreover, whilst mental disorders are strongly associated with suicidal behaviour worldwide, mood disorders are the strongest predictor of suicide ideation and attempts in developed countries and suicide is among the leading causes of death (Fawcett et al., 1990; Nock et al., 2008; Nock et al., 2009). MDD is more common in women than in men, although the gender difference is decreasing as gender roles become more equable in society (Seedat et al., 2009). Depressive disorders affect individuals across all age groups, including children (Merikangas et al., 2010) and the aged (Byers et al., 2010; Kessler, Birnbaum, et al., 2010).

In the majority of cases, depression shows a high rate of recurrence and substantial chronicity, indicating that the disorder is frequently not treated adequately (Gonzalez et al., 2010). Even in a primary care setting, only about one-quarter of identified patients with MDD achieved and maintained full remission for 18 months, whilst another quarter failed to remit at all. The remaining patients suffered either from residual symptoms or recurrences during follow-up (Vuorilehto et al., 2009). Randomised clinical trials show some efficacy of several types of interventions, including cognitive behavioural therapy or selective serotonin reuptake inhibitors (Gelenberg et al., 2010). However, given the substantial percentage of subjects who do not respond to treatment or who relapse after treatment discontinuation (Gelenberg et al., 2010), a better understanding of the psychopathology and pathophysiology, and their bi-directional interaction, of depressive disorders is essential. In turn, this should facilitate new and more efficacious preventative and therapeutic treatments (Belmaker & Agam, 2008).

Major depressive disorder presents as a disorder of feelings, thoughts and somatic functions that debilitate daily functioning and, as stated above, can be life threatening. The symptoms are heterogeneous and include emotional, cognitive and somatic dysfunctions that are used to make a nosological diagnosis. As for other mental disorders, two nosological classification systems exist for depression, and these are the Diagnostic and Statistical Manual of Mental Disorders (DSM), 4th edition, text revision (DSM-IV, 1994) and the International Classification of Diseases (ICD), 10th edition, chapter V: Classification of Mental and Behavioural Disorders (ICD-10, 1994). Both DSM and ICD recognise several forms of depressive disorder and grades of severity within these forms. According to DSM, MDD is the most common form of depression, and an approximate equivalent of MDD in ICD is (recurrent) depressive episode. According to DSM, MDD will be diagnosed if the clinical course is one or more major depressive episodes, with each such episode characterised by five (or more) symptoms during a minimum 2-week period, where at least one of the symptoms is either depressed mood (sadness, emptiness) or anhedonia (loss of interest or pleasure in (almost) all activities). According to ICD-10, (recurrent) depressive episode will be diagnosed if the clinical course is one (or more) episodes during a minimum 2-week period of at least two of three typical/core symptoms i.e. depressed mood, loss of interest and enjoyment, reduced energy leading to increased fatigability and diminished activity, and at least three (preferably four) common symptoms.

Based on describable and observable symptoms, the DSM and ICD nosologies allow for relatively clear diagnosis of and communication about MDD. However, the clinical entity MDD is not based on its neuropsychopathology, i.e. the changes in psychological processes, brain circuitry, and inter-cellular and intra-cellular brain functions, which causally underlie the symptoms of MDD. As such, there is incompatibility between the current diagnostic system and psychological and neurobiological research that would aim to increase understanding of MDD neuropsychopathology and, based on this, could lead to development of novel, improved treatments. Commensurate with this problem, some integration of the focus on specific symptoms and their underlying pathology — a dimensional diagnostic approach — is under consideration for the upcoming revision of the DSM (Hyman, 2007). Clearly, integration and translation across a number of disciplines in the clinical, social and biological sciences is needed to achieve the goal of a neuropsychopathology-based system of diagnosis and treatment in psychiatry.

The core/typical symptoms of depression can be viewed as disrupted emotional–motivational–cognitive states. Emotions e.g. sadness, helplessness, grief, relief, pleasure, are distinct psychological states that vary in intensity and that arise in response to environmental stimuli or events that are either aversive or rewarding, as processed by the brain's punishment system and reward system (Rolls, 2000). The punishment system and reward system are the bases of emotions, and emotions are the bases of mood; therefore, the punishment and reward systems are also the bases of mood. Cognitive processes enable the individual in her/his emotional categorisation of environmental stimuli/events, and also allow an environmental event to be re-experienced, or ruminated on, in the absence of its physical presence. The MDD core symptom of depressed mood therefore constitutes dysfunction(s) in the brain's punishment system, that manifests as chronic hyper-sensitivity to aversive events and is associated with symptoms/states such as sadness, pessimism, helplessness (Elliott et al., 2002). The MDD core symptom of reduced interest and pleasure constitutes dysfunction(s) in the brain's reward system that manifests as chronic hypo-sensitivity to rewarding events (Haber & Knutson, 2010). In this sense, MDD shifts from an absolute, abnormal emotional–cognitive nosological entity, to several emotional–cognitive states that are each at the extreme end of their continuous distribution (Hyman, 2007).

Emotional–cognitive assessment of environmental stimuli/events is not absolute, neither within individuals across time nor between individuals. For each individual, the emotional response to a stimulus/event is determined by: his/her alleles for genes that regulate emotional responsiveness (Pezawas et al., 2005); the expression levels of these alleles and their products in the specific brain regions that contribute to emotional–cognitive circuits (Choudary et al., 2005); and his/her life history with respect to prior emotional experiences (Caspi & Moffitt, 2006; Jacobs et al., 2006). Thus, the extent to which an aversive stimulus

activates the punishment system and elicits an emotion such as sadness or helplessness, or the extent to which a rewarding stimulus activates the reward system and elicits an emotion such as pleasure, will depend on current and prior experience as determined by genetic and environmental factors (Berridge & Robinson, 2003; Cools et al., 2007). Given that these same genetic and environmental factors determine personality, then personality is logically also a major predictor of emotional reactivity. Mood or mood state refers to the general emotional experiences, and therefore disposition, of an individual at a typical time point within a certain time period e.g. day, week, month.

The viewpoint has been presented above that MDD constitutes a psychopathology of chronically altered emotional–motivational–cognitive processing of aversive and/or rewarding environmental events, and that genetic and environmental factors are aetiological in the neuropsychopathology of MDD. It is of course striking that one or more environmental events can impact mental functioning to the extent that a healthy individual subsequently develops symptoms of emotional–cognitive disorder. Coming now to the major focus of this review, it is essential to ask: What are the environmental events that are most salient to MDD aetiology and neuropsychopathology, and why? It is of course a substantial challenge to study life events that occur in the contexts of e.g. employment, finance, housing, health, social relationships, with the aims of identifying which of these events are relevant to MDD and, if relevant, what their salient features are (Agid et al., 2000; Monroe & Reid, 2008).There is strong evidence that aversive/adverse life events occurring during development (e.g. childhood abuse, neglect) (Green et al., 2010; Kessler, McLaughlin, et al., 2010) and/or adulthood (Brown et al., 1995; Kendler et al., 2002; Monroe & Reid, 2008; Kendler & Gardner, 2010) are associated with increased vulnerability to MDD and that aversive/adverse environmental events proximate to the onset of MDD can trigger the disorder. The adverse life event categories identified in one major MDD study (Keller et al., 2007) were: death of a loved one, ending of a romantic relationship, personal failure or abandoned goal, chronic stress (due to work, finances, legal problems, etc.), own health problems, interpersonal conflict between self and other, and distress over future events. In this study, the different adverse life events predicted different MDD symptoms: for example, death of loved ones and romantic breakups were marked by high levels of sadness, anhedonia and appetite loss; chronic stress and personal failure were associated with fatigue and hypersomnia. It has been proposed that the most MDD-relevant aversive/adverse life events involve threat, loss or humiliation; are very threatening or unpleasant; are experienced by the person directly; and are of a distinct onset i.e. acute (Brown et al., 1995; Monroe & Reid, 2008). If such events are associated with MDD, then they have typically occurred within 3–6 months prior to MDD onset. With regard to childhood adversities, the strongest associations with (onset and persistence of) a broad spectrum of mental disorders, including mood disorders, are found with: maladaptive family functioning such as parental mental illness, substance abuse disorder, and criminality; family violence; physical abuse; sexual abuse; and neglect (Green et al., 2010; McLaughlin et al., 2010). Therefore, it might well be that the current mood of an individual can be linked to one or more identifiable major emotional events in the past, and that this is dominating emotional processing in the present.

A further characteristic of aversive/adverse environmental events that has been proposed to be central to MDD aetiology and psychopathology is the uncontrollability of the event. Thus, with respect to aetiology, if an environmental event is aversive and uncontrollable, it will perhaps be more likely to contribute to the aetiology of MDD than an event that is equally aversive in every other respect but, indeed, controllable. For example, in one major study (Kendler et al., 2003) life events that involved major loss, humiliation or entrapment predicted MDD. Whilst it is not possible to isolate and independently study the contribution of the uncontrollability of these life-event dimensions, uncontrollability is certainly a major characteristic of each of them. In the laboratory, it is possible to manipulate the (un)controllability of an aversive event, and studies doing so have demonstrated that uncontrollability per se is a major determinant of behaviour, leading to the theory of the learned helplessness effect (Seligman et al., 1971), the central theme of this review. If an individual is exposed to a real uncontrollable aversive life event of major emotional significance and consequently develops a generalised emotional–cognitive state of perceiving their environment as uncontrollable, then this state would appear to be central to the onset and maintenance of MDD. Indeed, this would be the emotional–cognitive state of (feelings of) helplessness and hopelessness, as frequently reported by MDD patients (Beck & Steer, 1988). In clinical psychology and psychiatry, the concepts of helplessness and hopelessness have been prominent in theories of depression and its treatment, as exemplified by the various psychometric instruments that have been developed to assess them.

In summary, the study of discrete emotional–cognitive states in MDD can provide essential data and insights with respect to the aetiology, pathophysiology and neuropsychopathology of the disorder and, therefore, to its treatment. Whilst helplessness is not a symptom according to current nosological classification, the learned helplessness effect is a neuropsychological process potentially of high importance to MDD aetiology, and helplessness is an emotional–cognitive state potentially of high importance to MDD neuropsychopathology.

### 1.2. Rationale for this review

The learned helplessness effect/helplessness constitutes the most prominent example of where an emotional–cognitive psychological concept has been proposed to be relevant to understanding and treating MDD. Learned helplessness (LH) has been proposed to be a major aetiological process in vulnerability to and onset of MDD (Seligman, 1972), and helplessness has been proposed as a major psychopathological mechanism in maintenance of MDD (Abramson et al., 1978). Originally, the LH effect was used as an explanatory variable for animal behaviour in the laboratory, and was subsequently translated to human behaviour, both in healthy circumstances and in MDD (Seligman, 1972). We are not aware of any systematic review that aimed to integrate LH/helplessness with concepts that are central to current psychiatry, including adverse life event aetiology, state markers and dimensional diagnosis. Given that a major advantage of the dimensional approach is that it is commensurate with translational (inter-species) research, and given that LH is a proven translational concept, then it would appear to be logical, important and timely to provide such a review. It should be comprehensive, considering LH with respect to animal psychology and neurobiology; animal modelling relevant to MDD; human neuropsychology; and MDD and emotional–cognitive state markers of MDD. Such a review should facilitate assessment of the utility of LH/helplessness with respect to increasing understanding of MDD aetiology, pathophysiology, neuropsychopathology and treatment response. Given the conceptual and translational importance of LH/helplessness in the study of MDD, then LH/helplessness should also be a focus of translational research aimed at discovery, validation and development of novel therapies for MDD. To the best of our knowledge, there has been no systematic review of either the evidence for the effects of antidepressants and other pharmacological classes on LH in animals, or for the effects of antidepressants and psychotherapy on helplessness in MDD patients. Such a comparative systematic review would provide insights into the potential utility of helplessness as a dependent variable in antidepressant discovery and development.

### 1.3. Objectives of this review

The first major objective of this article is to present an integrative overview of the theory and evidence for the psychology and neurobiology of LH/helplessness in laboratory rodents (rats, mice), healthy humans and

humans with depression. The second major objective is to present the findings of systematic reviews of publications on the following themes: LH in rats and mice in terms of the laboratory paradigms used and whether LH was demonstrated; effects of pharmacological interventions on LH in laboratory rodents; randomised control trials into effects of pharmacotherapeutic interventions, psychotherapeutic interventions, or combined pharmaco-/psycho-therapeutic interventions, on helplessness in MDD patients. Based on the findings of the first two objectives, the third objective is to clarify the current status of LH/helplessness as a translational research concept in MDD, and to make recommendations for future research strategy via which the study of helplessness can make a significant contribution to MDD research and to discovery and development of improved antidepressant therapies.

## 2. Integrative overview of the learned helplessness effect, helplessness and depression

### *2.1. Protocols, theory and evidence for the learned helplessness effect and helplessness*

#### *2.1.1. Rodents*

*2.1.1.1. The learned helplessness effect paradigm.* Solomon, Seligman, Overmier and Maier were studying animal operant learning, and the development of theories which could account for how animals learn associations between their behaviour and the occurrence of rewarding or aversive events (e.g. the two-process theory of avoidance learning, Rescorla & Solomon, 1967). Iconoclastically, they focused on the contingency where the probability that an aversive event will terminate following any behaviour is exactly equal to the probability that the same event will terminate in the absence of any behaviour. This, as Seligman and colleagues emphasised, is the response-aversive event contingency of uncontrollability (or the zero-operant contingency) (Fig. 1**A**) (e.g. Overmeier & Seligman, 1967; Maier & Seligman, 1976). For example: if the aversive event is an electro-shock (e-shock) administered to a rat in a 2-way shuttle box and the rat can always terminate the e-shock (i.e. escape) by moving from the side of the box it is occupying to the opposite side, then the rat will learn this contingency and exhibit such shuttling (escape) behaviour in response to e-shock. Now, if the contingency is that the termination of the e-shock is externally controlled, there will be no predictable relationship between any behaviour in the rat's repertoire (e.g. shuttle, jump, turn) and the termination of the e-shock. That is, the rat experiences uncontrollability of an aversive environmental event. Seligman and colleagues observed that experiencing uncontrollability of aversive events at one point in time and in one situation leads to animal subjects behaving in the same or a new situation as if there was still no response-aversive event contingency. The important difference was, though, that in this second experimental phase there was indeed a contingency i.e. escape responses were now possible but were not exhibited (Fig. 1A). Furthermore, even if escape responses were exhibited they did not lead to the learning of consistent escape responding. This observation was described as the learned helplessness effect.

The term LH effect is anthropocentric as applied to animal behaviour: helplessness is an emotional feeling that can be ascribed and quantified in humans on the basis of their verbal or written responses to questions. It cannot be ascribed on the basis of behavioural observation in humans and this is also true for animals, of course. Indeed, the term interference effect (e.g. Anisman & Merali, 2001) is more objective. Nonetheless, one of the major strengths of the LH effect was — and compared to many paradigms used currently, most certainly still is — the robust, unambiguous and objective manipulation used to induce it. Animal subjects are assigned at random to the treatment groups. The durations of the aversive stimuli experienced by the "no aversive control (NAC) group" are determined by and equal to the latencies to make escape responses to these stimuli exhibited by the "aversive control (AC) group". Using this so-called yoked design, the number, intensity and duration of the aversive

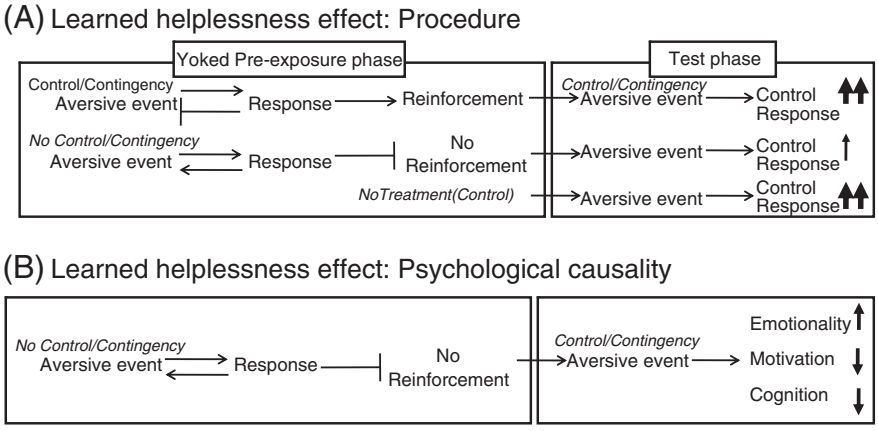



**Fig. 1.** Schematic presentation of the learned helplessness effect proposed by Seligman and colleagues, in terms of (**A**) experimental procedure and (**B**) causality at the psychological level. (**A**) The LH effect is demonstrated using a two-phase experimental design. The pre-exposure phase involves dividing subjects at random into three groups, aversive control (AC, referred to in the case of e-shock studies as escapable e-shock, ES), no aversive control (NAC, inescapable e-shock, IS) and Naive to aversive stimuli (Naïve, no e-shock, NS). The AC and NAC groups are exposed to a series of aversive events/stimuli, typically electro-shock, each of which has a maximum duration e.g. 5 s or 30 s. The AC subjects are able to terminate each aversive event via a specific operant behaviour, typically an active motor response. The AC subjects acquire the appropriate response via reinforcement, learning the contingency between behaviour and e-shock termination, with the relief of the latter the presumed major emotional response and major incentive-motivational factor. The latency to perform AC to each e-shock determines the duration of the e-shock presented to the paired (yoked) NAC subject. NAC subjects cannot terminate the e-shock; no specific response is reinforced any more (or any less) than any other response. On the day following the (last) pre-exposure session the test phase is conducted: all subjects are exposed to aversive events/stimuli, typically e-shocks, and are able to terminate each aversive event via a specific operant behaviour, typically an active motor response. Subjects in the AC and Naive groups exhibit active motor responses and acquire and exhibit the operant response that terminates (controls) aversive events. Subjects in the NAC group exhibit reduced active motor responses and do not acquire and exhibit the operant response that terminates (controls) aversive events. (**B**) The psychological causality of the LH effect is proposed to comprise three components, emotional, motivational and cognitive. AC subjects acquire the control response via reinforcement, with the relief of e-shock termination the presumed major emotional response and the major incentive-motivational factor. NAC subjects learn the absence of both contingency between responding and e-shock termination and of reinforcement. Any relief of e-shock termination is independent of behaviour. This could increase the aversive valence of the e-shock and the emotional response to it and to the environment in which it occurs. This could lead to reduced incentive-motivation to engage in behaviour that might terminate e-shock. The deficit in control responses is expressed during the test phase, suggesting that these cognitive, emotional and motivational effects pertain beyond the situation that induced them. Nonetheless, they could have specificity with respect to the circumstances in which they are exhibited and to longevity. Therefore, learned aversive uncontrollability (LAU) might be a more parsimonious descriptor than LH, with a chronic LAU being a necessary pre-requisite of generalised LH.

stimuli experienced are identical in the two groups. Accordingly, the reduced number and/or the increased latency of escape responses exhibited by the NAC group in the test phase of the experiment can be attributed to the prior experience of uncontrollable aversion specifically (Fig. 1**A**). That is, the LH effect is a model for the study of the effects of uncontrollability of aversive events on subsequent behaviour with respect to events that are controllable but are not perceived as such.

A further important characteristic of the animal experiments used by Seligman and colleagues to demonstrate the LH effect was the triadic design. That is, in addition to the AC and NAC groups there is a third group, the "naive to aversive stimuli (Naive) group", which is pre-exposed to the context without the explicit aversive stimuli (Fig. 1**A**). The Naive group is essential in order to control for the possibility that animals in the AC group are acquiring a specific form of escape behaviour in the context of the first (pre-exposure) phase and then using this to perform escapes in the second (test) phase. If this were the case, then the Naive group would behave similarly to the NAC group. As demonstrated by Seligman and colleagues, and subsequently by other groups, this is not the case and the Naive group exhibits a high level of escape behaviour comparable to the AC group, with the NAC group exhibiting a deficit relative to both of these groups. That is, the subjects in the AC group exhibit natural escape responding during the first and second phases of the LH protocol and the NAC group cannot do so during the first phase and consequently does not do so during the second phase. The triadic design is essential where the LH effect is induced in one situation (e.g. wheel turning is the operant that terminates/fails to terminate tail e-shock) and tested in a different situation (e.g. two-way shuttling is the operant that terminates paw e-shock). If the same situation (e.g. two-way shuttling) is used for both phases then the LH effect cannot be attributed to specific learning during the first phase and the Naive group becomes redundant (in effect the Naive group would be a second AC group). It is justifiable, therefore, to study the LH effect without the Naive group under these conditions. However, it is not possible to study the LH effect without the AC group, i.e. by comparison of an NAC group with a Naive group. As expected, such a comparison does yield an escape deficit in the NAC group: this deficit is described as the US pre-exposure effect (Randich & LoLordo, 1979) and is fundamentally different to the LH effect because it is not measuring the effect of aversive uncontrollability, the central tenet of the LH concept. As illustrated by the two systematic reviews of animal studies reported on in this paper, the US pre-exposure design has been used in many studies and is often inaccurately reported as the LH design (see Appendix A Fig. A1).

*2.1.1.2. Psychological causality of the learned helplessness effect.* As an explanation for the LH effect at the psychological level, Seligman and colleagues proposed that three intervening processes underlie the development of the deficit in the control response as exhibited by the NAC group; namely, emotional, motivational, and cognitive (Fig. 1**B**) (Maier & Seligman, 1976). The emotional process in the LH effect is an increase in negative emotion, inferred from observations that NAC rats exhibit altered behavioural repertoires and physical status during the test phase and in the home cage. These include increased stereotypy, decreased appetitive discrimination, altered feeding behaviour, and increased ulceration and heart rate. The motivational process is a reduced motivation to escape, inferred from observations in the test phase that NAC rats exhibit a reduced physical response to the aversive stimulus (e-shock) relative to the AC and Naive rats. That is, it is not the case that NAC rats are exhibiting the same level of physical activity during e-shock as are AC rats but that this is non-directional and therefore does not result in escape. Rather, the motor response is reduced, and could indicate a deficit in incentive-motivation to escape. The cognitive LH process is reduced ability to detect an association between an adaptive behavioural response and the consequences of that response. This was inferred from the findings that NAC rats do exhibit some escape responses, even at the beginning of the test phase, but do not exhibit a learning curve, i.e. escape responses do not become more frequent as a consequence of escape responding as the escape test progresses. Given that rodents exhibit goal-directed behaviour (Toates, 1986; Balleine & Dickinson, 1998), then it might be that this deficit is due to development of reduced expectancy that behavioural responding will exert predictable, reinforcing effects on the environment (Fig. 1**B**).

Alternative explanations have been proposed for the LH effect that would, if supported by the evidence, certainly question the relevance of the LH effect to the translational study of the inter-relationships between uncontrollability, helplessness and MDD. For example, it has been proposed that the deficit in the control response exhibited by the NAC group reflects a psychomotor deficit i.e. escape responding in the test phase requires a vigorous motor response of which NAC subjects are not physically capable. Evidence purported to support this interpretation of the LH effect includes the demonstration that NAC mice exhibit reduced locomotion in an open field test as well as a two-way escape deficit in the LH test phase (e.g. (Anisman et al., 1978a)). Of course, even if the deficit in the control phase is due to a motor deficit, this still begs the question of why this is specific to the NAC group, given that the amount of aversive stimulus pre-exposure received is equal to that received by the AC group. It is also important to note that psychological and psychomotor explanations are not mutually exclusive, particularly given that psychomotor retardation is a common symptom of MDD (DSM-IV, 1994).

Altered nociception dependent on the controllability of painful stimuli applied in the pre-exposure phase has also been proposed as an explanatory variable: uncontrollable e-shocks have been proposed to lead to an analgesic effect relative to identical e-shocks that are controllable (Whitehouse et al., 1985). Such an effect would be problematic given that the control response deficit in NAC subjects would then be due to reduced sensitivity to the aversive stimulus during the test phase relative to AC counterparts. Using independent measures of pain sensitivity, e.g. the hot plate test, to study effects of e-shock on nociception has not yielded evidence that mice exposed to e-shocks exhibit reduced nociception (Chourbaji et al., 2005). If pain sensitivity is reduced by uncontrollable painful stimuli, then hyper-activity in the endogenous opiate system would be a candidate mediating mechanism. As reviewed below (Section 3.3.3), pharmacological studies do not provide strong support for this interpretation, with sub-chronic morphine administration actually increasing escape behaviour in the NAC group.

*2.1.1.3. From learned helplessness effect to generalised helplessness?.* Therefore, the robust and observable LH effect in rodents might be mediated by a complex constellation of psychological processes: When an aversive event occurs it causes a heightened state of negative emotionality. The subject is motivated to terminate this aversive state and any behaviour that does so will result in relief and be reinforced and therefore repeated, based on the expectation that the aversive event can be controlled (Maier & Seligman, 1976). If the subject learns that its behaviour cannot control the aversive event then the negative emotionality will persist, the expectation that the aversive event can be controlled will decrease, and the motivation to control it will decrease, as will the abilities to learn that the contingency has changed and to acquire the expectation that the aversive event can be controlled. Accepting the evidence for the LH effect, and the explanation that it has emotional, motivational and cognitive components, it is essential to consider the inter-relationships between the LH effect in rodents and (i) a state of helplessness in rodents, and (ii) the LH effect, helplessness and MDD, in humans. We discuss inter-relationship i here and the inter-relationships ii in the next section.

The classical design of LH experiments in rats involves exposing subjects to aversive stimuli in one environment and testing for aversive-stimulus escape behaviour in a different environment (see section

3.2.2.1). Typically, the form of the aversive stimulus will be similar in both phases/environments, e.g. painful e-shock, but the nature of the stimulus delivering the aversive stimulation will be different, e.g. metal rod on tail at pre-exposure phase versus grid floor at test phase. Maier and Watkins (2005) refer to this set-up and the demonstration of the LH effect with it, as trans-situationality. Indeed, they propose that such trans-situationality provides evidence that the LH effect is generalised. Furthermore, they argue that the processes mediating trans-situational LH and within-situational LH could well be different (Maier & Watkins, 2005). For example, fear conditioning to context could be more important in within-situational LH. Whilst the demonstration of the trans-situational LH effect certainly provides evidence for a psychological state that is generalised, this state is also temporary, with a duration of several days maximum (Maier & Watkins, 2005).

A further issue of generalisation is whether the LH effect persists when the form or intensity of the stimulus used in the test phase is altered. For example, e-shock amplitude could be greater at test than at pre-exposure. This example has been investigated, with the finding being that NAC rats exhibit similar levels of escape behaviour to AC rats i.e. no LH effect (Jackson et al., 1978). This indicates that the LH effect can be specific rather than generalised. Another approach has been to compare operant behaviour for reward in NAC versus AC subjects and this has provided evidence for a generalised deficit: The paradigm used was signalled-punishment suppression of operant behaviour for reward, in which a tone CS signalled periods when subjects would be punished for operant responding. The NAC rats exhibited reduced CS-suppression of responding, suggesting a cognitive deficit in learning a contingency between their behaviour and aversive outcomes. One caveat with respect to interpreting this finding as evidence for generalised helplessness is that the aversive stimulus in both the LH effect and the signalled punishment experiments was the same i.e. e-shock (Jackson et al., 1978; Pryce & Seifritz, 2011). It will be important to test additional forms of aversive stimuli in LH subjects to try and increase the robustness of an interpretation of generalised helplessness.

The extent to which an LH effect extends to a generalised helplessness effect is very likely to depend on the severity of the aversive stimulation during the pre-exposure phase. As can be seen from the studies listed in Tables 2 and 3 (Section 3.1), there is marked variation in the duration and intensity of aversive pre-exposure deployed across studies: e.g. the wheel-turn tail e-shock paradigm uses 30-s maximum duration and two-way paw e-shock often uses 5-s maximum duration. There could well be a positive association between severity of aversive stimulation and extent of generalised helplessness developed. However it also needs to be emphasised that the more severe the aversive stimulus is for the NAC group then the more severe it is for the AC group also. A severe aversive stimulation, e.g. long latency until escape possible in the AC group, during the pre-exposure phase might lead to a statistical LH effect but a generalised helplessness effect might then pertain in both the NAC and AC groups, such that the aversive stimulation per se and not its controllability would be responsible, thereby rendering the study irrelevant to the relationship between uncontrollability, helplessness and depression.

Finally, it is important to note that, in addition to the LH effect, NAC animals could exhibit additional states of translational relevance to depression symptoms, such as weight loss, sleep disturbance, anhedonia. If this is the case, then it could well constitute strong supportive evidence for a generalised depression-relevant state that includes, and is also causally attributable to, the LH effect (see Discussion).

*2.1.1.4. Translational validity of the learned helplessness effect.* The validity of the LH effect in rodents as a translational model of relevance to MDD is, of course, dependent on the relevance of human LH and helplessness to MDD, and these issues are discussed next. According to the validity criteria proposed for animal models of human mental disorders (Willner, 1984; Markou et al., 2009), the rodent LH effect has high validity as a model for a human LH effect: There is aetiological validity with respect to aversive uncontrollability being the unambiguous causal factor in both rodents and humans. There is construct validity with respect to changes in emotion, motivation and cognition due to experiencing uncontrollable aversive event(s) appearing to underlie, at the psychological level, the perception of future events as uncontrollable, in both rodents and humans. There is face validity with respect to a deficit in goal-directed operant responding to controllable aversive events being the major dependent variable in both rodents and humans.

As noted above, the extent of the translational validity of the LH effect in rodents to the study of human MDD depends not only on the robustness of the LH effect, and of its generalisation to a helplessness state, but also on the relevance of a human LH effect and human helplessness to MDD risk, onset and maintenance. Consideration of these inter-relationships in humans needs to be carried out in a step-wise manner, by providing evidence relevant to the following questions: Can the LH effect be demonstrated in healthy humans using laboratory paradigms? Is the experience of uncontrollable aversive events a major aetiological factor in MDD onset? Do MDD patients exhibit increased helplessness relative to healthy control probands in laboratory LH paradigms? Is assessment of the general controllability of events reduced in MDD and, if yes, does this contribute to the maintenance of MDD? Is there evidence that the emotional, motivational and cognitive mediating processes proposed for the animal LH effect also pertain in humans and particularly so in MDD patients? Is reduction of helplessness therapeutic in MDD?

We now proceed to a concise review of the evidence pertinent to the above questions on human LH effect–helplessness–MDD.

### 2.1.2. Humans

*2.1.2.1. Laboratory learned helplessness paradigms.* The two-phase (aversive pre-exposure, aversive test) × three group (AC, NAC, Naive) paradigm designed to investigate the LH effect in animals has been translated directly into laboratory paradigms for the study of the LH effect in healthy humans. These studies have been reviewed elsewhere (e.g. Hiroto, 1974; Abramson et al., 1978; Miller & Norman, 1979) and will be summarised here, in terms of design, findings and interpretation. As in animal LH effect paradigms, either the same or a different aversive stimulus has been used in the pre-exposure phase and the test phase, in human LH effect studies (Fig. 1**A**). Aversive stimuli used have been either a painful noxious stimulus such as an e-shock to the finger or heat shock to the wrist, a noxious loud noise, or a non-noxious high-demand cognitive task. Each of these stimulus types has been used at the pre-exposure phase and/or the test phase, in various combinations. At the pre-exposure phase, the AC group is given either a noxious escapable stimulus, e.g. press a button to terminate an e-shock, or a solvable cognitive task. The NAC group is given, respectively, either a noxious inescapable stimulus, the duration of which is yoked to the escape latency of the AC group, or an unsolvable cognitive task, and the Naive group is not given any stimuli. At the test phase, all groups are given either a noxious escapable stimulus or a solvable cognitive problem and the level of their control responding is measured. Typically, both the inability to escape noxious stimulation or to solve cognitive problems in the NAC group, induces deficits in the test phase, on either escaping escapable noxious stimuli or solving solvable cognitive tasks, relative to the other two groups. With regards to the psychological process(es) mediating the LH effect, Seligman and colleagues have, as in animals, proposed that induction of a cognitive state of non-contingency between (own) behaviour and environmental events is a major mediating factor (Abramson et al., 1978). Other authors (e.g. Buchwald et al., 1978) have proposed that it is not necessary to infer non-contingency as a mediating mechanism; rather, uncontrollability at the pre-exposure phase is proposed to be responsible for failure at the test phase. However these authors have

not provided an alternative explanation in terms of what process mediates the observed effects of uncontrollability.

In addition to the 2 phase × 3 group design that is essential to study the LH effect, cross-over designs have also been used in humans to study the effects of aversive stimulus (un)controllability on task performance and on self-reports of feelings of control and helplessness. Such designs might require fewer subjects, and have typically been the designs of choice in studies that have compared effects of uncontrollability in MDD patients versus healthy probands. All subjects are studied across either two or three consecutive phases, i.e. either an initial NAC phase is followed by an AC phase (e.g. Bolz & Giedke, 1981), or an initial AC phase is followed by an NAC phase (i.e. withdrawal of control), which in turn is followed by a second AC phase (i.e. reinstatement of control e.g. Diener et al., 2009). The major outcome measures are: does the NAC phase result in a deficit in the AC phase; do subjects report reduced feelings of control during NAC; do subjects report increased feelings of helplessness during NAC. Studies using cross-over designs have typically not demonstrated a deficit in the control response (e.g. latency to button press to terminate e-shock) following reinstatement of AC but do nonetheless induce states of reduced controllability and increased helplessness as based on self-report (e.g. Diener et al., 2009). Furthermore, cross-over designs have demonstrated phase-specific differences in controllability and helplessness state between MDD and healthy subjects, as reported below (section 2.2.2).

*2.1.2.2. Helplessness as a consequence of uncontrollable life events.* In the laboratory, exposure to (un)controllable aversive events will typically lead to the LH effect in animals and humans (Maier & Seligman, 1976; Abramson et al., 1978). However, in animals and humans, generalised helplessness is certainly not a necessary consequence of uncontrollable real-life events. It has been proposed that uncontrollable outcomes will only be sufficient to induce generalised helplessness if the estimated probability of the occurrence of a highly aversive outcome is high or of the occurrence of a highly desired outcome is low (Abramson et al., 1978). As noted above (Section 1.1), life events that involve severe forms of loss, humiliation or entrapment that are beyond the individual's control do indeed predict MDD (e.g. Kendler et al., 2003).

*2.1.2.3. Learned helplessness theory of depression: original.* The LH effect was put forward as an aetiological explanation for animal behaviour and then human behaviour in a set of specific test conditions, and emotional, motivational and cognitive processes were invoked. The extent to which the LH effect should, if at all, be extrapolated to a learned helplessness theory of depression has been a matter of debate, even among the original proponents of the LH effect. Abramson, Seligman, and Teasdale (Abramson et al., 1978) argued that "the learned helplessness hypothesis claims that depressed affect is a consequence of learning that outcomes are uncontrollable" (p. 50). However, Maier and Watkins stress that the learned helplessness theory was not developed in order to provide an animal model of depression or of any other clinical condition: "Indeed, the word 'depression' did not appear in any of the original papers concerning this phenomenon, and did not do so for at least 7 years. The term behavioural depression was not used to describe stressor controllability phenomena till some 15 years later (…)." (p. 830) (Maier & Watkins, 2005). Furthermore, they argue that consequences of exposure to uncontrollable stressors are equally similar to symptoms of depression and symptoms of extreme anxiety, and sensitive to both antidepressants and anxiolytics (Maier & Watkins, 2005).

Nonetheless, during the past four decades, the LH effect has been extrapolated to a model of depression and to the LH theory of depression. In referring to the LH effect, Seligman observes: "Such uncontrollable events can significantly debilitate organisms: they produce passivity in the face of trauma, inability to learn that responding is effective, and emotional stress in animals, and possibly depression in man." (p. 407) (Seligman, 1972). Seligman stresses the multiple parallels between animal behaviour in the LH paradigm and features of depression in humans. Both animals in the LH paradigm and humans suffering from depression show "reduced response initiation as well as a 'negative cognitive set' — difficulty in believing or learning that one's own responses will succeed even when they do" (p. 411). He also alluded to neurobiological parallels between the two conditions (Seligman, 1972). Furthermore, already in the late 1960s, studies showed that through behavioural interventions it was possible to reverse or prevent learned helplessness in animals (Seligman & Maier, 1967; Seligman et al., 1968), and Seligman interpreted these findings as an analogue to prevention and intervention in humans (Seligman, 1972).

In sum, the LH effect as described in animals and humans was by some, and soon after its initial description, reformulated as a theory to account for the psychopathology of depression.

*2.1.2.4. Learned helplessness theory of depression: refined.* Approximately 10 years after its original formulation, the LH theory of depression underwent a major revision to embrace a more differentiated picture of cognitive processes related to helplessness in humans. This revision followed the general acceptance of cognitive processes as being of scientific interest in (clinical) psychology (often referred to as the 'cognitive turn') and drew on previous concepts, such as attribution theory (Heider, 1958; Rotter, 1966; Abramson et al., 1978). Some authors have stated that from the very beginning, the LH concept included a "cognitive" component, in that it postulated that mere exposure to uncontrollability is not sufficient to induce helplessness; rather the organism must come to expect that outcomes are uncontrollable (Abramson et al., 1978). Nonetheless, until the late 1970s, a differentiated picture of cognitive processes related to LH theory was lacking.

According to Seligman and colleagues, the need for the revision was based on three major problems when applying a LH theory to humans and depression: i) the theory did not distinguish between cases in which outcomes are uncontrollable for all people and cases in which outcomes are uncontrollable only for some people (universal versus. personal helplessness); ii) the theory did not explain when helplessness is specific and when it is general; and iii) the theory did not explain when helplessness is acute and when it is chronic (Abramson et al., 1978).

According to the revised helplessness theory, it is not necessarily low control over the environment itself, but rather how an individual subjectively perceives his control over a situation. How subjects attribute their current loss of control is argued to be the central process (Abramson et al., 1978). Once an individual perceives non-contingency, he/she attributes it to a cause. This cause can be stable or unstable, global or specific, and internal or external. Moreover, the individual will perceive helplessness as personal (the person expects the outcome is contingent on a response in the repertoire of a relevant other) or universal (the person expects the outcome is not contingent on a response in the repertoire of any relevant other). The attributions will influence whether expectation of future helplessness will be chronic or acute, broad or narrow, and whether helplessness will lower self-esteem or not (Abramson et al., 1978).

The LH theory of depression has since undergone several smaller specifications and refinements. Ten years after the Abramson–Seligman–Teasdale reformulation, another major extension of the LH theory of depression, the 'hopelessness theory of depression', was put forward (Abramson et al., 1989). This introduced hopelessness depression as a subtype of depression, stating that hopelessness is a sufficient cause of depression, deemphasising the relevance of causal attributions and instead emphasising inferred negative characteristics about the self (Abramson et al., 1989). Most empirical data have been supportive of such a hopelessness depression subtype (Spangler et al., 1993; Joiner et al., 2001).

Therefore, the LH effect provides an aetiological explanation for how experience of uncontrollable aversive stimuli leads to a deficit in control and invokes emotional, motivational and cognitive processes,

in animals and humans. The original LH theory of depression proposes that human depression is a cognitive deficit in perceiving an association between own behaviour and environmental events; it is not a theory of depression aetiology and does not invoke emotional or motivational processes. The refined LH theory of depression is a refinement of the description of the cognitive deficit, and is also not a theory of depression aetiology and continues to de-emphasise the relevance of emotional and motivational processes. Perhaps for the first time, therefore, Fig. 2 presents a hypothetical model that extends the LH effect to a learned helplessness hypothesis of depression aetiology, onset and maintenance, invoking emotional, motivational and cognitive processes.

*2.1.2.5. Helplessness rating scales and their relationship to major depressive disorder.* Numerous psychometric instruments have been developed to capture concepts that fall in the broad field of helplessness, such as causal attribution, locus of control or perception of control (Lefcourt, 1991). The Beck Hopelessness Scale (BHS) (Beck & Steer, 1988) is one of the most commonly used of these instruments worldwide. Using the BHS it has been shown that subjects diagnosed with depression show higher rates of hopelessness than unaffected individuals (Beck & Steer, 1988). Hopelessness is also related more generally to current state of health, perceived health, disability, and some socio-demographic variables (Hamzaoglu et al., 2010). Since the development of the revised LH theory of depression, numerous studies have confirmed the association between helplessness, attributional style and depressive disorders (Nolen-Hoeksema et al., 1992). Appropriately, the extent of causality in the association of helplessness attribution/feeling of hopelessness with depression has been a matter of debate. As an important indication that cognitions related to helplessness (assessed with an attributional style questionnaire) are at least markers of depression, Seligman and colleagues (Seligman et al., 1988) showed that in patients with unipolar depression treated with cognitive therapy, both explanatory style and depressive symptoms improved by the end of therapy; moreover, changes in explanatory style were correlated with change in depressive symptoms. However, as this study did not include a randomised intervention (experimental manipulation) component, conclusions about the causality between helplessness related cognitions and depression are not possible.

Taking the explanation proposed for the LH effect, that it is the product of emotional, motivational and cognitive effects of exposure to uncontrollable aversive stimuli, then helplessness should probably also be studied as a composite of these three processes, and not as a specifically cognitive domain. This might then lead to increased understanding of the role of helplessness in the aetiology of MDD and as a state marker of MDD (Pryce & Seifritz, 2011).

*2.1.2.6. Comparison of major depressive disorder patients and controls on laboratory LH paradigms.* As emphasised above, care needs to be taken whenever bringing together discussion of the LH effect with discussion of MDD, its aetiology, onset and maintenance. The cross-species demonstrations of the LH effect do not by any means constitute evidence that LH is involved in the aetiology, onset or maintenance of MDD, or that the psychological processes that underlie LH are also causally involved in MDD. At the same time it is pertinent to discuss experimental designs that would further understanding of the inter-relationships between the LH effect, helplessness and MDD. In this respect the obvious experiment is to apply the 2 phase × 3 pre-exposure group design to MDD versus healthy control subjects, with subjects allocated at random to their respective pre-exposure groups. This design and the outcome predictions are presented in Fig. 3. There are actually very few studies to-date that have used this design. Those that have were carried out primarily by Seligman and colleagues (e.g. Abramson et al., 1978). They provide supportive evidence for the outcomes depicted in Fig. 3. Namely, that healthy subjects pre-exposed to NAC exhibit, relative to healthy subjects pre-exposed to AC, a similar deficit in control response at test phase to depressed subjects pre-exposed to AC, whereas depressed subjects pre-exposed to NAC exhibit a marked deficit in control responding. However, in

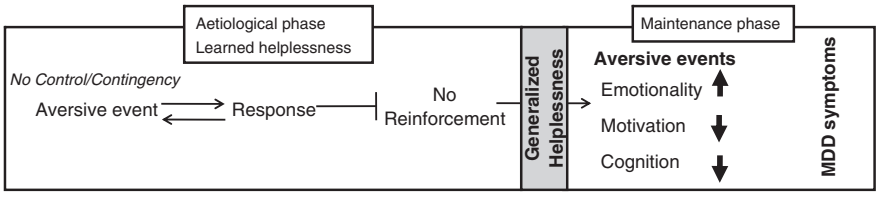



**Fig. 2.** Extension of the LH effect to an LH hypothesis of depression aetiology, onset and maintenance. The pre-exposure phase of the LH effect paradigm is replaced by an aetiological phase, where an uncontrollable aversive life event occurs and the individual experiences the uncontrollability as an absence of reinforcement in the form of failing to find a response that brings relief from the aversive event. A specific LH effect ensues. If sufficiently severe and chronic, the LH effect will induce pathophysiological processes that underlie onset of generalised helplessness. The generalised helplessness will include increased emotional reactivity to aversive but every-day stimuli, reduced motivation to engage these aversive every-day stimuli, and reduced cognitive expectancy that these aversive every-day stimuli are controllable. The generalised helplessness state will induce additional pathophysiological processes that induce (additional) symptoms of depression.

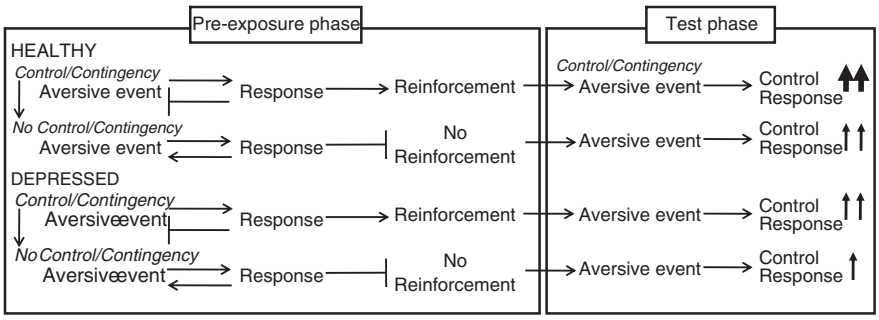



**Fig. 3.** Experimental design and predictions of outcome for comparison of depressed versus healthy proband performance in the LH paradigm. Healthy probands and depressed probands are both allocated at random to AC or NAC groups for the pre-exposure phase. In the test phase, all probands are exposed to an AC test, and the control response is measured. It is hypothesised that the amount of the control response will differ between the groups as follows: Healthy × Controllable > Healthy × Uncontrollable = MDD × Controllable > MDD × Uncontrollable. In addition to measuring the control response, it is also possible to assess subjects on a helplessness scale or a controllability scale at each stage of the paradigm. It is also possible to utilise the paradigm in an fMRI context.

these studies, the validity of the diagnostic status of the depressed subjects (typically, undiagnosed students who scored high on the Beck depression scale) and therefore the validity of the findings, have rightly been questioned (e.g. Buchwald et al., 1978).

There would appear to be a need for further such studies, therefore. Furthermore, test phase responses should be integrated with psychometric scores for controllability and helplessness, and the behavioural and psychometric measures should be intergrated with neurobiological studies, using functional imaging techniques.

### *2.2. Neurobiological regulation and correlates of the learned helplessness effect and helplessness*

#### *2.2.1. Rodents*

Given that the LH effect is likely mediated by emotional, motivational and cognitive processes and their complex interaction, the neurobiology of LH is also complex. Several substantive reviews of specific, major aspects of the neurobiology of the LH effect in rodents exist (Anisman et al., 1979; Weiss & Simson, 1986; Maier & Watkins, 2005). The current evidence for the brain regions, neurocircuits, neurotransmitters, neuropeptides and receptors involved is reviewed here briefly (and in Fig. 4**A**), but sufficiently to allow comparison with the human evidence derived from imaging studies, as reviewed below.

The neurobiology of acute responding to aversive stimuli and the evidence for chronic effects of aversive stimuli on neurobiology, per se, i.e. regardless of whether or not the stimuli are controllable, are obviously relevant to the neurobiology of the LH effect. With respect to brain regions involved in processing aversive stimuli, there is evidence, obtained primarily with the rat, for important involvement of: specific brain-stem nuclei including the dorsal raphé nucleus (DRN) and locus coeruleus (LC); the amygdala, periaqueductal grey (PAG), habenula, hippocampus and hypothalamus; and the prefrontal cortex (PFC), including the prelimbic (PL) region, infralimbic (IL) region and anterior cingulate cortex (ACC) (Millan, 2003). With respect to neurotransmitters, serotonin (5-HT) (Maier & Watkins, 2005; Ansorge et al., 2007), noradrenaline (NE) (Arnsten, 2009) and dopamine (DA) (Moghaddam, 2002) have important neuromodulatory effects on acute responding to aversive stimuli, and these monoamines and their receptors can be modified by chronic exposure to aversive stimuli (Duman et al., 1997). The major excitatory neurotransmitter glutamate and the major inhibitory neurotransmitter gamma-aminobutyric acid (GABA) mediate acute responses to aversive stimuli within and between regions (Moghaddam, 2002; Hashimoto, 2009), and their signalling functions are modified by chronic exposure to aversive stimuli, including via modified neuron-glia interactions (Sanacora et al., 2008). A number of neuropeptides and hormones, including corticotrophin releasing factor (symbol approved by the Human Genome Organisation Gene Nomenclature Committee is CRH) and corticosterone, are acutely and chronically increased by aversive environmental exposure (Koob, 1999; Maier & Watkins, 2005).

With respect to the neurobiology of stimulus (un)controllability, evidence has been obtained using various methods, including immediate–early gene (e.g. Fos) expression, lesioning, and pharmacological infusion. In mouse, Fos expression was compared in e-shock AC, NAC and Naive subjects, in amygdala, hypothalamus, LC and DRN. In each of these regions, Fos protein was increased in the NAC mice relative to the other two groups which were similar to each other, thereby indicating acute signalling of aversive uncontrollability per se (Liu, Tang & Sanford, 2009). Using lesioning, it has been demonstrated in rat that an intact DRN, during both pre-exposure and test phases, is necessary for NAC to lead to deficits in the control response (Maier et al., 1993). Processing of the (un)controllability of an aversive stimulus requires integration of somatosensory input indicating aversive stimulation onset/offset with somatomotor feedback indicating when a motor response has been made. The cortex is the primary region of such sensory–motor integration. The ventro-medial PFC (vmPFC) of the rodent brain is the analogue of the ACC of the human brain (Ongur & Price, 2000; Brown & Bowman, 2002). The two major regions of the rodent vmPFC are the PL and IL cortices, and, in rat at least, these two regions account for nearly all of the cortical input to the DRN (Gabbott et al., 2005). This input takes the form of long-range glutamatergic projections, which synapse on GABAergic interneurons in the DRN that project to 5-HT synthesising neurons of the ascending 5-HT system (Jankowski & Sesack, 2004). Under conditions of vmPFC activation, therefore, this circuitry exerts an inhibitory effect on 5-HT release from the DRN (Celada et al., 2001), that would perhaps counter-act the direct excitatory inputs to the DRN from regions such as the amygdala via, for example, synaptic CRH release (Mo et al., 2008; Magalhaes et al., 2010).

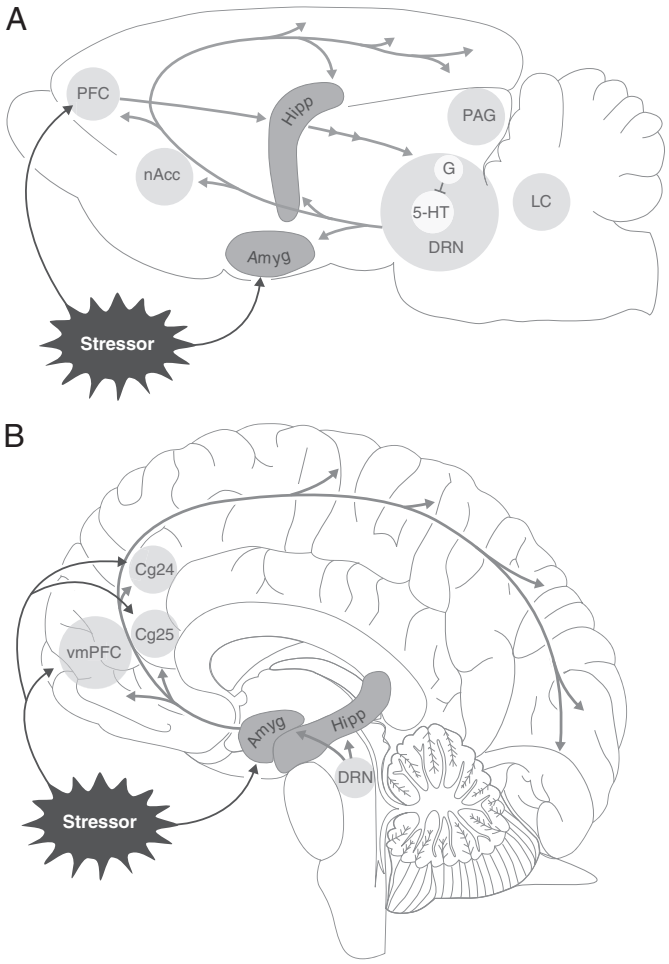



**Fig. 4.** Schematic representations of current evidence for (**A**) regulation of the learned helplessness effect in rat and (**B**) correlates of helplessness in human depression. (**A**) In rat there is evidence that the controllability of aversive stimuli is processed by and controls output from the ventro-medial prefrontal cortex (PFC). This PFC output is in the form of glutamate projections to GABA (G) interneurons in the dorsal raphé nucleus (DRN), and inhibits prolonged serotonin (5-HT) output. In the absence of inhibition of prolonged ascending serotonergic output to corticolimbic circuitry i.e. during periods of uncontrollable aversive stimulation, changes in this corticolimbic circuitry mediate the LH effect. Such changes presumably involve PFC assessment of controllable stimuli as uncontrollable. (**B**) In human there is evidence in healthy subjects that perceived state of uncontrollability is positively associated with activity in the anterior cingulate cortex (ACC: supragenual ACC, Cg24a, b; subgenual cingulate, Cg25), and in MDD patients that perceived state of helplessness is positively associated with ACC/PFC activity, and dysfunctional attitude is associated with 5-HT receptor changes in terminal regions, particularly in the PFC. Additional abbreviations: LC, locus coeruleus; PAG, periaqueductal gray; AMYG, amygdala; HIPP, hippocampus; nACC, nucleus accumbens. Copyright belongs to Sarah Steinbacher, SIVIC Scientific Visualisation and Visual Communication, University of Zurich, Zurich, Switzerland.

Using pharmacological infusion, it has been demonstrated in rat that vmPFC inhibition using the $GABA_A$ receptor agonist muscimol, followed by exposure to controllable aversive stimuli (AC group) leads to a subsequent test-phase deficit in the control response, i.e. as if these rats were in the NAC group. Furthermore, such rats exhibit a marked acute 5-HT response to the AC pre-exposure, again of a magnitude that would be typical of NAC pre-exposure (Amat et al., 2005). Conversely, excitation of the vmPFC via infusion of the $GABA_A$ receptor antagonist picrotoxin in rats then pre-exposed to NAC leads to control responding typical of AC pre-exposure (Amat et al., 2008). These rat findings suggest that the (un)controllability of an aversive stimulus is detected in/relayed to the vmPFC, and that controllability is positively associated with excitatory output from long-range glutamatergic projection neurons in the vmPFC to the DRN. Uncontrollability results in inhibition of vmPFC output and therefore a marked, acute 5-HT response and synaptic release in the ascending mesocorticolimbic pathway, including at the amygdala, hippocampus and PFC. How this acute response could lead to a chronic effect in terms of a deficit in the test-phase control response, also requires explanation of course. One possibility would be that high excitatory 5-HT input to the vmPFC, in synergy with other neuromodulators e.g. CRH, leads to a chronic increase in activity in GABAergic interneurons in the vmPFC (Tan et al., 2004) and thereby to a chronic "under-estimation" of the controllability of aversive stimuli i.e. the LH effect.

### 2.2.2. Humans

*2.2.2.1. Studies of neural correlates of aversive stimulus (un)controllability in healthy subjects.* A small number of studies have investigated neural activity in association with manipulations of the controllability of aversive stimuli in healthy subjects specifically. These studies provide insights into neural circuitry relevant to the LH effect. It is important to note that all of these studies used a cross-over design with aversive stimuli and that the sequence of (un)controllability used was the same for all subjects, thereby precluding the possibility to study the LH effect.

One of these studies reports on the neural correlates of controllability in terms of the post-imperative negative variation (PINV) of the electroencephalogram (EEG) (Diener et al., 2010). The paradigm comprised a warning tone (S1) that preceded a tone CS (S2) that was followed by a 1 ms e-shock to the index finger. Subjects were instructed to press a button as fast as possible when S2 was presented to avoid the aversive stimulus. There were three consecutive phases: control, loss of control and restitution of control. During the control and restitution of control phases, the aversive stimulus could be avoided and during the loss of control phase the aversive stimulus was uncontrollable in 50% of trials. Helplessness and controllability were measured on self-report scales of 0 to 100. The PINV was increased during the loss of control phase, when subjects reported feelings of low controllability and high helplessness. Using electromagnetic tomography, the source of the PINV increase during loss of control was localised to the anterior cingulate cortex (ACC).

Another study focused on neural responses to heat stimuli to the forearm, measured using functional magnetic resonance imaging (fMRI). Subjects were misleadingly informed that on some trials, indicated by a discriminatory visual stimulus, they could control the duration of the heat stimulus and that on other trials they could not. The impact of perceived uncontrollable pain on responding to controllable pain, i.e. the LH effect, was not investigated. When pain was perceived as uncontrollable, subjects exhibited increased activation in areas consistently linked with pain processing, namely secondary somatosensory cortex, ACC and insula (Salomons et al., 2004). In a follow-up correlational study (Salomons et al., 2007), neural activity was studied in relation to inter-individual differences in the impact of perceived controllability of the duration of the heat stimulus on self-reported pain perception. Some subjects reported higher levels of pain during perceived NAC versus perceived AC; these subjects exhibited greater activation in the pregenual ACC (pACC). Some subjects reported lower levels of pain during perceived NAC versus perceived AC; these subjects exhibited greater activation in the ventrolateral PFC (VLPFC) in the anticipatory period. These data indicate that the (perceived) controllability of pain stimuli impact on PFC activity, with uncontrollable pain associated with higher activity in the ACC (and insula) than controllable pain. During pain anticipation, uncontrollability is associated with relatively high activity in the VLPFC, and it has been proposed that high activity in VLPFC represents an attempt to regulate the emotional response to uncontrollable pain (Salomons et al., 2007).

Association between aversive stimulus controllability and frontal activity is also reported by Fretska et al. (1999). In the opposite order to that used to investigate the LH effect, subjects were first presented with solvable (induction of control) arithmetic tasks and then to unsolvable (withdrawal of control) such tasks. Using EEG to measure event-related slow cortical potential (SCP), when the tasks became unsolvable there was a positive neocortical potential shift (indicative of cortical activity reduction) for all neural regions except for fronto-polar regions. The latter exhibited negative potential change when the tasks became unsolvable; this is considered indicative of neuronal excitation and it was concluded that fronto-polar regions were engaged in the processing of uncontrollability. In a follow-up study using the same paradigm, Bauer et al. (2003) measured SCP in women and identified an activity decline localised in the ACC during the unsolvable condition. The decline was specific to a subgroup of female subjects who exhibited high de-motivation scores during the unsolvable task condition.

Each of the above studies identified the ACC as a region that is differentially activated depending on the perceived (un)controllability of an aversive stimulus (Fig. 4B). With various methods, the ACC exhibited increased activity during NAC, thereby implicating the ACC as an important candidate region in the mediation of the LH effect. However, discrepant results also need to be mentioned here. Using solvable and unsolvable anagrams and measuring regional cerebral blood flow (rCBF) changes using positron emission tomography (PET), rCBF was increased relative to baseline during unsolvable tasks in mamillary bodies and amygdala and decreased in hippocampus; during solvable tasks, rCBF was reduced in mammillary bodies and increased in hippocampus. Both conditions increased rCBF in frontal and temporal regions. The authors suggested that uncontrollability predominantly affected limbic areas relative to frontal or temporal regions (Schneider et al., 1996).

*2.2.2.2. Studies of neural correlates of aversive stimulus uncontrollability and helplessness in MDD and healthy subjects.* A systematic review was conducted of human studies that have compared depressive patients and controls, applying any form of neural activity, and either assessing helplessness and/or controllability and related variation of brain activity, or experimentally modifying controllability of a situation and investigating associated variation of neural activity. Out of 143 human studies identified by literature search and assessed for eligibility, nine studies fulfilled the inclusion criteria and were included in quantitative analysis. Characteristics of the studies included are depicted in Table 1. It is important to note that in all of the below studies that used a cross-over design with aversive stimuli, the sequence of (un)controllability used was the same for all subjects, thereby precluding the possibility to study the LH effect.

Diener et al. (2009) assessed PINV of the EEG in unmedicated depressed subjects and controls using the forewarned S1–S2 finger e-shock paradigm (described above). Task performance was similar in MDD and control subjects across the three phases. Controllability scores were similar between MDD and healthy subjects and were reduced during the loss of control phase. Helplessness scores were

higher in the MDD group compared to controls across all phases. In addition, whilst helplessness increased during loss of control and returned to control levels during restitution of control in healthy subjects, MDD subjects reported high helplessness during the loss of control phase and this persisted into the restitution of control phase. Depressed subjects showed relatively higher frontal PINV during the loss of control and restitution of control phases. The relatively high helplessness scores of MDD subjects, particularly during the restitution of control phase, indicate a lack of association between the (specific) task controllability measure and the (general) helplessness measure. It is noteworthy that persistence of both high helplessness and high PINV activity into the restitution of control phase characterised the MDD group. These results expand on previous findings of higher PINV in depressed patients compared to controls during NAC conditions (Bolz & Giedke, 1981). This study of Diener et al. also demonstrates that feelings of controllability over a specific aversive stimulus task and feelings of helplessness are not necessarily correlated in MDD (Table 1).

An fMRI study compared MDD and healthy subjects in terms of neural correlates of exposure to aversive painful stimuli (Strigo et al., 2008). The aversive stimulus was moderate heat pain to the forearm and was always uncontrollable (NAC), with a signalled anticipation phase preceding aversive stimulation. All subjects were unmedicated. During the anticipation of painful (NAC) stimuli, MDD subjects showed increased blood oxygen level-dependent (BOLD) activity in the right anterior insula and dorsal ACC compared to controls. A follow-up region-of-interest analysis of the amygdala showed increased right amygdala BOLD activity in MDD subjects compared to controls. In MDD subjects, right amygdala BOLD signal correlated positively with perceived helplessness as measured using a pain catastrophising scale during the anticipation phase. The findings suggest that increased helplessness in MDD is associated with abnormal amygdala activity.

In two other fMRI studies (Grimm, Boesiger, et al., 2009; Grimm, Ernst, et al., 2009) and one EEG study (Chiu & Deldin, 2007), all of which used the Beck Hopelessness Scale (BHS), correlations between hopelessness scores and frontal cortex activity were compared between MDD and control subjects (Fig. 4**B**). Again, these studies did not investigate the LH effect. Grimm, Boesiger, et al. (2009) investigated subject-group differences in negative BOLD response (NBR) during an emotional processing task, in which subjects were shown pictures and were required to judge the picture as negative or positive. The MDD subjects showed decreased NBR in the pregenual and supragenual ACC and the ventromedial PFC (VMPFC), compared to healthy controls. In the MDD group, low NBR was associated with high feelings of hopelessness. Using the same task, Grimm, Ernst, et al. (2009) showed that in MDD subjects the dorsomedial PFC (DMPFC) was activated less than in controls during emotional picture judgement, and that low DMPFC activation was associated with high BHS scores and also with scores on the Beck Depression Inventory (BDI).

Three PET studies describe correlations between 5-HT receptor availability (defined as binding potential, BP) and hopelessness levels measured using the Dysfunctional Attitudes Scale (DAS). DAS scores are considered to indicate negatively biassed views of oneself, the world, and the future, and DAS scores are correlated highly with BHS scores in MDD subjects. Two PET studies describe higher 5-$HT_{2A}$ receptor (5-$HT_{2A}$R) BP in both MDD and remitted MDD subjects compared to healthy controls (Meyer et al., 2003; Bhagwager et al., 2006). DAS scores were positively associated with 5-$HT_{2A}$R BP in several cortical regions in unmedicated MDD subjects (Meyer et al., 2003) and in frontal regions in remitted MDD subjects (Bhagwager et al., 2006). High 5-$HT_{2A}$R BP suggests receptor up-regulation in depression, and particularly so in subjects with high DAS scores, and might be a consequence of chronically impaired 5-HT release (Meyer et al., 2003). The third PET study (Meyer et al., 2004) reports an absence of differences between MDD and control subjects in 5-HT transporter (SLC6A4; alias 5-HTT) BP. However, within MDD subjects there were correlations between DAS scores and 5-HTT BP in brain regions that receive 5-HT projections, including the PFC, ACC, thalamus, caudate, and putamen. Overall the PET data indicate that those MDD subjects with more severe dysfunctional attitudes, suggestive of high hopelessness, show more severe changes in 5-HT terminal regions, particularly in the PFC (Fig. 4**B**).

Integrating the current human evidence above, it is noteworthy that in healthy subjects, perceived state of uncontrollability is positively associated with ACC activity, and in MDD patients perceived state of helplessness is positively associated with ACC/PFC activity. These findings suggest that the ACC is central to both the LH effect and the state of helplessness in MDD, and could therefore be important in mediating any causal relationship between these two processes (Fig. 2). Furthermore, in the rat, the brain region which is analogous to the human ACC is central to mediation of the LH effect. Future translational studies can build on this comparative evidence, to establish a detailed neuro-psychological understanding of the LH effect, helplessness and MDD, and their inter-relationships.

**Table 1**
Overview of studies that assessed correlates of brain function in relation to controllability and helplessness in MDD patients and controls.

| Reference | Method/Helplessness-related measure | Subjects |
|---|---|---|
| Bhagwager et al., 2006 | PET | 7 female/13 male controls |
| | Dysfunctional Attitudes Scale | 8 female/12 male remitted MD subjects |
| Bolz & Giedke, 1981 | EEG | 10 female/8 male controls |
| | | 10 female/8 male subjects with MD |
| Chiu & Deldin, 2007 | EEG | 17 controls |
| | Beck Hopelessness Scale | 18 subjects with MD |
| Diener et al., 2009 | EEG | 14 female/12 male controls |
| | Controllability and Helplessness measures | 16 female/8 male unmedicated subjects with MD |
| Grimm, Boesiger, et al., 2009 | Functional MRI | 21 female/8 male controls |
| | Beck Hopelessness Scale | 11 female/8 male subjects with MD |
| Grimm et al., 2009 | Functional MRI | 12 female/13 male controls |
| | Beck Hopelessness Scale | 9 female/16 male subjects with MD |
| Meyer et al., 2004 | PET | 10 female/10 male controls |
| | Dysfunctional Attitudes Scale | 11 female/9 male subjects with MD |
| Meyer et al., 2003 | PET | 5 female/11 male controls |
| | Dysfunctional Attitudes Scale | 5 female/11 male unmedicated subjects with MD |
| Strigo et al., 2008 | Functional MRI | 10 female/5 male controls |
| | Controllability measure and Pain | 12 female/3 male unmedicated subjects with MD |

## 3. Systematic reviews of rodent studies of the learned helplessness effect, rodent studies of drug effects on the learned helplessness effect, and human studies of therapeutic intervention efficacy on helplessness in major depressive disorder

### 3.1. Methods of the rodent and human systematic reviews

#### 3.1.1. Criteria for including studies in the reviews

Three sets of inclusion criteria were applied, one for each of the rodent reviews (Triadic design, Pharmacology), and one for the human review (randomised controlled trial (RCT)).

*3.1.1.1. Rodent.* Triadic design: all randomised studies using a triadic yoked design comparing the effects of three types of aversive stimulus pre-exposure on behaviour were considered for inclusion in this review: controllable (AC; escapable stress, ES), uncontrollable (NAC; inescapable stress, IS), no pre-exposure (Naïve; no stress, NS).

Pharmacology: all randomised studies using a triadic yoked design (AC, NAC, Naive) or a dyadic design (NAC, Naive) to investigate the effect of a pharmacological, vehicle-controlled intervention after the pre-exposure phase on test phase behaviour were considered for inclusion in this review.

*3.1.1.2. Human.* All randomised controlled human studies comparing measures of helplessness (see below) between subjects with MDD receiving active treatment and subjects receiving either another active treatment or a control intervention or no intervention (e.g. wait-list control group), were considered for inclusion in this review. The exposure had to consist of any intervention aimed at treating depression or reducing symptoms of depression (active treatment), which was compared with either another active treatment, a control intervention (e.g. placebo or another intervention not including the assumed active component of the active treatment intervention), or no intervention (e.g. wait-list control group). Case reports were excluded.

#### 3.1.2. Subjects

*3.1.2.1. Rodent.* Triadic design and Pharmacology: all studies using post-pubertal rats (genus: *Rattus*) or mice (genus: *Mus*) were included.

*3.1.2.2. Human.* We included all studies involving adult (≥18 years) subjects that fulfilled the criteria for a diagnosis from the category of depressive disorders, according to DSM-IV; or depressive episode or recurrent depressive disorder or dysthymia, according to ICD-10, regardless of any comorbid mental disorders. The diagnosis had to be established through a diagnostic interview or by above cut-off scores in an established depression scale (e.g. Center for Epidemiological Studies Depression Scales (CES-D) (Radloff, 1977), Beck Depression Inventory (Beck et al., 1961), Hamilton Rating Scale for Depression (Hamilton, 1960)), indicating the presence of (mild, moderate, or severe) depression.

#### 3.1.3. Outcome measures

*3.1.3.1. Rodent.* Triadic design and Pharmacology: outcomes of interest were animal behaviour assessed by behavioural measures considered to be indicative of helplessness or deficit in controllability/contingency, analysed as primary or secondary outcome. These included escape behaviour, lever pressing, freezing, immobility.

*3.1.3.2. Human.* Outcomes of interest were changes in levels of helplessness, hopelessness or locus of control, as assessed by a diagnostic instrument (questionnaire, interview) and analysed as primary or secondary outcome. Information extracted and reported comprises: i) changes from pre- to post-treatment in helplessness measures in the active treatment group(s) and control group(s); ii) differences in helplessness measures between the active treatment and comparison group at post-treatment, and; iii) differences between the active treatment and comparison group regarding change from pre- to post-treatment in helplessness measures.

#### 3.1.4. Search strategy for identification of studies

*3.1.4.1. Rodent.* Triadic design and Pharmacology: first, we searched Embase and Medline via the web-based advanced search and retrieval system provided by Embase (http://www.embase.com/search/advanced), from 1975 (for Embase) or 1966/1950 (for Medline/Old Medline, respectively) up to July 2010, using the web-browser Firefox. We applied the following string that excludes any type of review, moreover restricting our search to articles on animals and articles published in English: (((helplessness OR uncontrollab* OR controllab* OR "escape failure") AND (rat OR rats OR mouse OR mice)) NOT [review]/lim NOT [conference review]/lim"). Second, we searched Psycinfo via the OvidSP search and retrieval system provided by Ovid Technologies Inc. (http://ovidsp.ovid.com/ovidweb.cgi?T=JS&MODE=ovid&PAGE=main&NEWS=n&DBC=y&D=psyh) from 1806 up to July 2010, via the web-browser Firefox, and using the field-search option. We restricted our search to articles on animals and articles published in English, and excluded any type of review. We combined (AND) the following two strings (using the calibrations: "Key Concepts": id, "Subject Heading": sh, "Tile": ti, "Abstract": ab, "Heading Word": hw, "Original Title": ot, and "Publication Type": pt): 1. (helplessness or uncontrollab: or controllab: or "escape failure"), 2. (rat or rats or mouse or mice).

Combining the search in the different databases and removing duplicates led to 1314 hits. The articles identified by this search were assessed according to the criteria of consideration, and separately for the Triadic design and Pharmacology reviews. There was no contact with authors. Furthermore, we hand-searched bibliographies of the ten most-recent original publications identified and evaluated them according to the criteria of consideration for this review outlined above. The flowcharts of the study inclusion procedure for the searches Triadic design and Pharmacology are provided in Appendix A Fig. A1.

*3.1.4.2. Human.* Information on the use of scales assessing helplessness or related constructs are usually not primary study outcomes within RCTs and hence often not reported in the title, abstract or key words. Therefore, we used Google Scholar in order to be able to perform a full-text search. We searched Google Scholar on August 7th 2010 from Switzerland, via the web-based search and retrieval system provided by Google Inc. (http://scholar.google.com), using the web-browser Firefox, selecting "Google Scholar in English" and articles (unselecting the inclusion of patents) with the search term:

("efficacy scale" OR "helplessness subscale" OR "control scales" OR "I-E scale" OR "helplessness scale" OR "control scale" OR "hopelessness scale") AND (intitle:depressive OR intitle:depression OR intitle: depressed) AND "randomised controlled") We restricted our search to articles providing at least summaries, but did not place any restriction on the date.

The search revealed 166 hits. The publications identified by this search were assessed according to the criteria of consideration for this review outlined above. There was no contact with authors. The flowchart of the study inclusion procedure for RCT is provided in Appendix A Fig. A2.

#### 3.1.5. Selection and coding of the data

*3.1.5.1. Rodent.* Triadic design: One investigator (DA) reviewed all articles. If there was ambiguity in selection or coding, DA and CRP discussed this issue and resolved disagreements until they reached consensus. We identified 43 published studies from the last 42 years that met our inclusion criteria (Appendix A Fig. A1). The extracted data included the species, type of pre-exposure aversive stimulus, apparatus used to apply the aversive stimulus, type of aversive test stimulus, nature

of the control response, apparatus used to assess the control response, indicator/measure of the control response, and main results regarding behavioural differences in control responding between study groups.

Pharmacology: one investigator (DA) reviewed all articles. If there was ambiguity in selection or coding, DA and CRP discussed this issue and resolved disagreements until they reached consensus. We identified 15 published studies from the last 33 years that met our inclusion criteria. The extracted data included the species, type of pre-exposure aversive stimulus, apparatus used to apply the aversive stimulus, pharmacological class and drug/compound name, type of aversive test stimulus, nature of the control response, apparatus used to assess the control response, indicator/measure of the control response, and main results regarding: i) differences in control responding between the pharmacologic and vehicle groups at test phase, and; ii) differences between the NAC (IS) group and comparison group(s) with respect to differences in control responding between the pharmacologic and vehicle groups.

*3.1.5.2. Human.* Two investigators (MT and GM) reviewed all articles independently. If there was ambiguity in selection or coding, these investigators discussed this issue and resolved disagreements until they reached consensus. We identified 10 published studies, all from the last 7 years, that met our inclusion criteria. The extracted data included patient diagnosis, diagnostic instruments, types of treatment and control intervention, target construct and applied scale to measure target construct, number of patients included, and main results, including: i) changes from pre- to post-treatment in helplessness measures in the active treatment group; ii) differences in helplessness measures between the active treatment and comparison group at post-treatment, and; iii) differences between the active treatment and comparison group regarding change from pre- to post-treatment in helplessness measures.

### 3.1.6. Assessment of heterogeneity

*3.1.6.1. Rodent.* Heterogeneity of studies was assessed with regard to relevant study characteristics, including rat/mouse strain, type, intensity and number of aversive stimuli in the pre-exposure stage, paradigm used to assess the control response, and sample size.

Triadic design: in sum, there was substantial heterogeneity of the studies in several key aspects (see Sections 3.2.2.1, 3.2.2.2), including statistical procedures. The mean number of animals was 10 per group (median: 10, range: 8 to 17).

Pharmacology: in sum, there was substantial heterogeneity of the studies in several key aspects (see Results section 3.3.3), including statistical procedures. The mean number of animals was 11 per group (median: 10, range: 6 to 14).

*3.1.6.2. Human.* Heterogeneity of studies was assessed with regard to relevant study characteristics, including diagnosis, intervention, measure of helplessness, and sample size. In sum, there was substantial heterogeneity of the studies in several key aspects (see Results section 3.4.3). The median number of subjects was 54 (range: 32 to 267).

As previously suggested, in case of substantial heterogeneity across studies, pooled estimates should not be published (Blettner et al., 1999). Even though several procedures exist that allow for quantitative synthesis in spite of heterogeneity, we refrained from applying them, as in our case their caveats (Ioannidis et al., 2008) would have outweighed the advantage of producing summary scores with questionable explanatory power.

## 3.2. Systematic review of rodent studies of the learned helplessness effect

### 3.2.1. Study selection

Since the original rat experiments of Seligman and colleagues a number of studies of the effects of aversive stimuli on subsequent escape behaviour have been conducted in rats and mice. A systematic review of these studies was conducted, with randomised studies that used the triadic yoked design to compare the effects of three types of aversive stimulus exposure (AC (ES), NAC (IS), Naive (NS)) on subsequent operant behaviour, being the criteria for inclusion. Appendix A Fig. A1 presents a flowchart of the literature search. Out of 1314 studies identified by literature search and assessed for eligibility, there were 38 rat publications and six mouse publications that fulfilled the inclusion criteria and were included in quantitative analysis. The great majority of studies did not use the triadic design but rather the dyadic design of NAC versus Naive and therefore the LH effect could not be measured.

### 3.2.2. Synthesis of results

*3.2.2.1. Rat.* The rat findings are presented in Table 2. Firstly, it is interesting to note the publication year of the 38 relevant publications: 5% were published in 1960–69, 18% in 1970–79, 34% in 1980–89, 21% in 1990–99, and 21% in 2000–2009. That is, the number of publications has remained consistent since the initial description of the LH effect. Thirty six of the studies were conducted with male rats only, one with males and females, and only one study focused on females. The 38 relevant publications present a total of 48 experiments. In a minority of experiments (26%), a tone or light was used as a conditioned stimulus (CS) to predict the aversive unconditioned stimulus (US), either in the pre-exposure phase, test phase, or in both phases. These experiments are discussed separately below. The most commonly used aversive stimulus during the pre-exposure phase is paw e-shock (58%), the second most common is tail e-shock (31%), and the remaining experiments (10%) use water, either immersion under water or forced swimming. Different forms of apparatus are used to present and control paw e-shocks. The most common is an operant set-up in which rats in the AC group terminate e-shocks by pressing a lever (35%) and the second most common set-up is a 2-way shuttle box where the e-shock can be stopped by AC rats crossing from one compartment to the other (15%). The next most common set-up is an operant box using a platform to jump onto or a pole to climb up, to terminate paw e-shocks, in the AC group (8%). All tail e-shock experiments are performed in a wheel-turn arena in which the e-shock can be terminated in the AC group by the rat turning the wheel.

For the test phase in rats, paw e-shocks are the escapable stimulus in 67% of studies. A number of apparatus types are used to present these e.g. 44% of all studies use a 2-way shuttle box to present paw e-shocks, 15% use a lever operant box. Tail e-shock is not used at all as the aversive stimulus in the test phase. Six experiments use water paradigms, three involving swimming duration in a forced swim test (Weiss et al., 1981; Brown et al., 2001; Drugan et al., 2005) and the other three escape via a ramp (Braud et al., 1969) or via a guillotine door (both experiments in Altenor et al., 1977). In the remaining experiments, no aversive US is used, and the test takes the form of elevated plus maze (Grahn et al., 1995; Korte et al., 1999) or context-conditioned freezing (e.g. Rosellini et al., 1987; Baratta et al., 2007). With respect to the combination of pre-exposure and test phases used, two groups prevail: paw e-shock in lever press operant box followed by paw e-shock two-way shuttle box is used in 10 experiments (21%), and tail e-shock wheel-turn followed by paw e-shock two-way shuttle box is used in seven experiments (15%).

The integration of classical conditioning into the experimental design, as used in ten experiments, involves a tone or a light CS predicting the aversive US. In those studies deploying such CS-US conditioning, it is used in the pre-exposure phase only (e.g. Korte et al., 1999), in the test phase only (e.g. Baratta et al., 2007), or in both phases (e.g. Bersh et al., 1986). In some cases the CS predicts the escapable US and in other cases the CS can itself be escaped, therefore allowing US avoidance (e.g. Korte et al., 1999). Some experiments use different CSs in the two phases (Cotton et al., 1982; Cotton & Smith, 1990). In some experiments in which the same CS is used in both phases, the US

**Table 2**
Overview of studies that used the IS-ES-NS design to test for the learned helplessness effect in rat.

| Reference | Pre-exposure phase | | | Test phase | | |
|---|---|---|---|---|---|---|
| | Stressor | Apparatus | Escape response | Stressor | Apparatus | Measure |
| LH effect demonstrated | | | | | | |
| Volpicelli, 1983, 1984 | Foot electro-shock | 2-way shuttle box | Locomotor transfer | Foot electro-shock | Operant arena w/o CS | Lever press = shock termination |
| Leuner, 1994 | | | | Aversive CS | Eyeblink conditioning chamber CS (light), US (periorbital shock) | Eyeblink expression to CS |
| Altenor et al., 1977, Blustein, 1992[a]; Hemingway & Reigle, 1987, Wellman, 1998; Whitehouse et al., 1985 | | Operant arena | Lever press | Foot electro-shock | 2-way shuttle box w/o CS | Locomotor transfer = escape |
| Follick, 1981, Mauk, 1979 | | | | Foot electro-shock | Operant arena w/o CS | Lever press/chain pull = shock termination |
| Seligman, 1975b | | | | | Activity arena with platform | Jump onto platform = escape |
| Rosellini et al., 1987, Warren, 1988 | | | | | Activity arena with platform | Avoid grid floor |
| Telner & Singhal, 1981 | | Activity arena with platform | Jump on to platform | Foot elector shock | 2-way shuttle box w/o CS | Locomotor transfer = escape |
| Hannum, 1976, Seligman, 1975a | | | | Foot electro-shock | Operant arena w/o CS | Lever press/chain pull = shock termination |
| Braud et al., 1969 | | Arena | Pole-climbing | Water exposure | Water tank | Swimming to target = escape |
| Korte et al., 1999 | Foot electro-shock with CS (tone, light) | 2-way shuttle box | Locomotor transfer (escape/avoid) | | Elevated plus maze | Time in open arm |
| Ilin & Richter-Levin, 2009 | | | | Foot electro-shock | 2-way shuttle box | Freezing expression to context |
| Ilin & Richter-Levin, 2009 | | | | | w/o shock, with CS | Freezing expression to CS |
| | | | | | | Locomotor transfer=escape CS |
| Cotton et al., 1982; Cotton & Smith, 1990 | | Operant arena | Lever press | Foot electro-shock | 2-way shuttle box with different CS | Locomotor transfer = Avoid /escape |
| Bersh et al., 1986; Bersh et al., 1990 | | Operant arena | Lever press | Foot electro-shock | 2-way shuttle box w/o CS | Locomotor transfer = Avoid /escape |
| Bersh et al., 1986 | | | | Foot electro-shock | 2-way shuttle box with CS | Locomotor transfer = Avoid /escape |
| Amat 2001, 2005, Blake-Mortimer, 1998, Hellhammer, 1984, Maier, 1977, 1988 | Tail electro-shock | Operant arena | Wheel turn | Foot electro-shock | 2-way shuttle box w/o CS | Locomotor transfer = Escape |
| Glazer, 1976 | | | | Foot electro-shock | Operant arena w/o CS | Lever press = shock termination |
| Glazer, 1976 | | | | Foot electro-shock | Arena with barrier with CS (tone) | Jump barrier = Avoid /escape |
| Amat et al., 2005, Baratta et al., 2007 | | | | Aversive context | Operant arena w/o CS | Freezing expression to context |
| Baratta et al., 2007 | | | | Aversive CS | Operant arena with CS | Freezing expression to CS |
| Rozeske, 2009 | | | | Aversive context | 2-compartment-box with shock | Conditioned place preference |
| Kelsey, 1977 | Tail electro-shock with CS (Tone, Light) | Operant arena | Wheel turn (only escape) | Foot electro-shock | 2-way shuttle box with different CS | Locomotor transfer = Escape |
| Weiss et al., 1981 | Tail electro-shock with feedback signal | Operant arena | Wheel turn | Water exposure | Forced swim test | Immobility |
| Altenor et al., 1977 | Underwater exposure | Water tank | Door escape | Electro foot-shock | 2-way shuttle box w/o CS | Locomotor transfer = escape |
| Drugan et al., 2005 | Water swim stress | Operant water tank | Lever press | Water exposure | Force swim test | Immobility |
| Brown et al., 2001 | | | | Electro foot-shock | 2-way shuttle box w/o CS | Locomotor transfer = escape |
| LH effect not demonstrated | | | | | | |
| Shors, 2007[b] | Foot electro-shock | 2-way shuttle box | Locomotor transfer | Electro foot-shock | 2-way shuttle box w/o CS | Locomotor transfer = escape |
| Altenor et al., 1977[c] | | Operant arena | Lever press | Water exposure | Underwater maze | Swimming to target = escape |
| Ragusa, 1969 | | | | Electro foot-shock | 1-way runway apparatus | Locomotor transfer = escape |
| Grahn et al., 1995 | Tail electro-shock | Operant arena | Wheel turn | Aversive context | Elevated plus maze | Time in open arm |
| Altenor et al., 1977[c] | Underwater exposure | Water tank | Door escape | Water exposure | Underwater maze | Swimming to target = escape |
| Brown et al., 2001[c] | Water exposure | Water tank with lever | Lever press | Water exposure | Forced swim test | Immobility |

[a] Study conducted with females
[b] Study conducted with females and males.
[c] Same study as above showing LH effect, in which a different experiment did not obtain an LH effect.

(e-shock) is renounced in the test phase such that CS-motivated freezing or 2-way avoidance responding is measured under extinction conditions (Ilin & Richter-Levin, 2009). It is interesting to assess whether or not inclusion of a CS impacts on the magnitude of the LH effect. Very few of the studies identified used a design where analysis of the CS per se on the LH effect was the aim of the study. In one such study, rats were pre-exposed to nine daily sessions of paw e-shocks that were paired on between 50% and 100% of trials with a tone CS. Only in those rats where the CS-US contingency was 100% and only when the CS was also used at the test phase, was the LH effect observed (Bersh et al., 1986). This and related studies (e.g. Bersh et al., 1990) provide evidence that CS-US conditioning can be essential to or increase the LH effect under specific conditions of AC-NAC pre-exposure. A gross overview of the studies cited in Table 2 does not yield any qualitative evidence that, under the experimental conditions typically used, an LH effect is more or less likely to be obtained by using a CS at pre-exposure and/or test phase; a reliable assessment of quantitative effects can only be assessed within study.

Of the 48 rat experiments using the triadic design, 42 (88%) report the LH effect: the NAC (IS) group exhibits a significant reduction in performance of the control response during the test phase relative to the AC (ES) and Naïve (NS) groups, with the latter two groups exhibiting similar, high levels of control responses (Table 2). Of the six experiments that did not demonstrate the LH effect, there is no consistent characteristic of the paradigm used in either the pre-exposure phase or the test phase that could provide a methodological explanation for the lack of effect.

*3.2.2.2. Mouse.* The mouse findings are presented in Table 3. Six mice studies fulfil the search criteria for the triadic design. Five of these are performed in males only and one in males and females (Anisman et al., 1978b). Only one study has been performed since 2000 (Palermo-Neto et al., 2003). In each of these studies, paw e-shock is used in the pre-exposure phase: four studies are conducted with a 2-way shuttle box (including one study that also used a CS) and two studies with a lever operant box. The test phase comprised escapable paw e-shock in four studies: two in a shuttle box (Anisman et al., 1978b; Anisman et al., 1980), one in an activity arena with platform (additional experiment in Anisman et al., 1978b), one in a lever press operant box (Caspy & Lubow, 1981) and one in a free-choice T-maze (Anisman et al., 1984). One study uses water in the test phase with latency to climb onto a ramp to escape being the dependent measure (Caspy et al., 1979), and one uses the elevated plus-maze and the total distance moved on it (Palermo-Neto et al., 2003). There is a single study applying a CS during the pre-exposure phase, during which the escape group can avoid or escape the CS-paw e-shock pairings (Palermo-Neto et al., 2003). All six studies demonstrated the LH effect, with the NAC (IS) group exhibiting a significant reduction in performance of the control response during the test phase relative to the AC (ES) and Naive (NS) groups, with the latter two groups exhibiting similar, high levels of control responses (Table 3).

### *3.3. Effects of pharmacological treatments on the learned helplessness effect in rodents*

#### *3.3.1. Study selection*

A systematic review was conducted of all randomised studies that used a triadic yoked design (AC, NAC, Naive) or a dyadic design (NAC, Naive) to investigate the effect of a pharmacological, vehicle-controlled intervention on the relationship between the type of pre-exposure and behaviour in the test phase. Appendix A Fig. A1 presents a flowchart of the literature search. With respect to the timing of the pharmacological manipulation, the aim was to review the studies where the intervention follows the pre-exposure i.e. therapeutic design. Such studies can provide insights into the pharmacological reversal of the LH effect, and therefore have translational predictive relevance. With respect to the design of the pre-exposure manipulation, the primary interest was to study the effects of pharmacological treatments on the LH effect; that is, the effect on the deficit in the control response exhibited by the NAC group relative to the AC group. The systematic review identified two drug studies that used such a triadic design and the remaining drug studies used the NAC versus Naive comparison only. The latter design is problematic because it is unclear whether any control response deficit in the NAC group is due to prior exposure to aversive stimulation generally or to aversive uncontrollability specifically. However, given that the studies were conducted using pre-exposure + test phase paradigms in which the LH effect is robust, then the Naive group provides a reasonable surrogate for the absent AC group. Of major interest was whether the pharmacological effect on test-phase behaviour was specific to the NAC group, i.e. in statistical terms was there a Pre-exposure group x Drug interaction effect, with the Drug effect specific to the NAC group?

Out of 1314 rodent publications identified by literature search and assessed for eligibility, 15 fulfilled the inclusion criteria.

#### *3.3.2. Study characteristics*

The characteristics of these studies are presented in Table 4. Fourteen of the studies were conducted in rat and one in mouse (Anisman et al., 1980). As noted above, two of these studies were based on the triadic design (Whitehouse et al., 1985; Hemingway & Reigle, 1987) and the remainder on a dyadic design. In the rat, the most commonly used aversive stimulus during the pre-exposure phase was paw e-shock (80% of studies), the second most common was tail e-shock (13%). For the test phase the 2-way shuttle box (69%) and the operant box with lever press (31%) were the arrangements used. In the 2-way shuttle box, six of the eight studies used a CS to predict the paw e-shock in the test phase.

The 15 publications report on a total of 33 experiments (Table 4). These experiments investigate the effects of several different drug classes, namely antidepressants, neurotransmitters, catecholamine receptor agonists or antagonists, endogenous opioid receptor antagonist or agonist, a NMDA-receptor antagonist, a benzodiazepine

**Table 3**
Overview of studies that used the IS-ES-NS design to test for the learned helplessness effect in mouse.

| References | Pre-exposure phase | | | Test phase | | |
|---|---|---|---|---|---|---|
| | Stressor | Apparatus | Escape response | Stressor | Apparatus | Measure |
| Anisman et al., 1978a, 1980[a] | Foot electro-shock | 2-way shuttle box | Locomotor transfer | Foot electro-shock | 2-way shuttle box w/o CS | Locomotor transfer = escape |
| Anisman et al., 1978b | | | | Foot electro-shock | Activity arena with platform | Jump onto platform = escape |
| Anisman et al., 1984 | | | | Foot electro-shock | T-maze with footshock | Correct discrimination response |
| Caspy et al., 1979 | Foot electro shock | Operant arena | Lever press | Water exposure | Water tank | Ramp escape |
| Caspy & Lubow, 1981 | Foot electro-shock | Operant box | | Lever press = escape | | |
| Palermo-Neto et al., 2003 | Foot Electro-shock with CS (tone/light) | 2-way shuttle box | Locomotor transfer (escape/avoid) | Aversive context | Elevated plus maze | Open arm entries |

[a] Study conducted with females and males.

receptor agonist and a nitric oxide synthase inhibitor. The treatment is acute (0–60 min pre-test phase), sub-chronic (5 days pre-test phase) or chronic (14 days pre-test phase). The treatment was administered either systemically (intra-peritoneal, subcutaneous, oral) or infused into a specific brain region using stereotactic methods. In five of the experiments (e.g. Danchev et al., 1989) the drug was administered to NAC group only, so it is not possible to determine whether any drug effect was specific to the subjects that were exhibiting a deficit in the control response. That it is possible that drug effects are not group-specific is demonstrated by the study of Harrell et al. (1978), in which it is reported that acute administration of the α1-adrenoceptor agonist aramine increased the control response in the test phase in both the NAC and Naive groups (Table 4).

*3.3.3. Results of individual studies and synthesis of results*

The studies of effects of existing antidepressant drugs are especially important given that these provide evidence on the predictive validity of the LH effect as a "model of depression". Eight experiments were conducted with antidepressants: six with a tricyclic, namely desipramine (four), imipramine or nortriptyline, and two with the selective serotonin reuptake inhibitor (SSRI) zimelidine (Table 4). Four of these experiments report that the deficit in test-phase control responding in the NAC x vehicle group was reversed by drug, with the drug treatment being acute nortriptyline (Telner & Singhal, 1981), acute desipramine infused into the dorsal hippocampus (Joca et al., 2006), acute desipramine infused into the frontal neocortex (Sherman & Petty, 1980), and acute zimelidine infused into the dorsal hippocampus (Joca et al., 2006). In the other four antidepressant experiments there is no evidence for an effect of drug treatment.

Such a (acute) drug effect in the NAC group specifically, i.e. reversal of the LH effect, is also reported for several other drugs. With respect to neurotransmitters, GABA infused into the hippocampus or the lateral geniculate body reversed the NAC group deficit (but not in several other regions e.g. frontal cortex, septum, amygdala) (Sherman & Petty, 1980). Serotonin infused into the frontal neocortex or the septum reversed the NAC effect (but not in several other regions e.g. hippocampus, lateral geniculate body, amygdala, nucleus accumbens) (Sherman & Petty, 1980). Interest in exogenous opioid agonists is in part related to the clinical evidence that they are therapeutic in MDD: the mu-opioid receptor agonist morphine, after sub-chronic treatment, increased the control response in NAC rats specifically (Besson et al., 1996). In the same study, sub-chronic treatment with the mu-opioid receptor antagonist naloxone reduced the control response in both groups (Besson et al., 1996). In contrast to this latter finding, another study of naloxone, using an acute treatment at a high dose, reversed the NAC effect (Hunziker, 1992). Furthermore, in a triadic-design study with another mu-opioid receptor antagonist, naltrexone, administered acutely, the LH effect was also reversed (Whitehouse et al., 1985). Acute naloxone at low doses did not impact on the LH effect (Hemingway & Reigle, 1987; Hunziker, 1992). The rationale for these opioid antagonist studies is the hypothesis that painful stimuli such as e-shocks might increase analgesia when they are uncontrollable, which in turn leads to the deficit in the control response, and hyper-activity in the endogenous opiate system mediates this analgesia (Whitehouse et al., 1985) (See Section 2.1.1.2 for a discussion of this issue).

As reviewed above (Section 2.2.1), exaggerated excitation of 5-HT neurons in the DRN during NAC pre-exposure has been proposed as an important mediator of the LH effect in rats. In relation to this evidence, the nitric oxide synthatase inhibitor Nw-nitro-L-arginine methyl ester (L-NAME) was microinjected into the DRN, on the basis that glutamate induced nitric oxide formation within the DRN might contribute to the LH effect via protracted effects within this nucleus: indeed, treatment with L-NAME did reverse the LH effect (Grahn et al., 2000). This finding is difficult to reconcile with the evidence that disinhibition of vmPFC glutamate signalling prevents the LH effect (see Section 2.2.1). More consistent with the latter evidence is the finding that antagonising DRN NMDA glutamate receptors using the antagonist 2-amino-5-phosphonovaleric acid (APV) did not reverse the LH effect ((Grahn et al., 2000); Table 4). As reviewed above (Section 2.1.1.2), it has been hypothesised that the LH effect can be accounted for in terms of a motor deficit that is mediated by depletion of DA and NE specific to subjects that experienced NAC. In an attempt to test this hypothesis, in mice, the DA receptor agonist morphine and the NE receptor agonist clonedine were tested: in support of the hypothesis, acute treatment with either morphine or clonedine reversed the LH effect (Anisman et al., 1980).

A number of the other findings summarised in Table 4 need to be highlighted. In addition to those anti-depressant experiments that did not demonstrate a reversal of the NAC effect, there was also no evidence for a therapeutic-like effect with several other drug classes. Noradrenaline infused specifically into each of the following regions was without effect: frontal neocortex, septum, hippocampus, lateral geniculate body, entorhinal cortex, posterior neocortex, caudate, nucleus accumbens, amygdala (Sherman & Petty, 1980). No evidence was obtained for therapeutic effects of clinical anxiolytics on the NAC effect: thus, neither sub-chronic treatment with the antihistamine hydroxyzine nor the benzodiazepine diazepam increased control responding in the NAC group (Porsolt et al., 1989). A modulatory role for noradrenaline in the DRN in the mediation of the LH effect has been proposed: however, infusion into the DRN of the α1-adrenoceptor antagonist benoxathian prior to the test phase did not affect the NAC effect (Grahn et al., 2002). There are additional Pre-exposure group x Drug interaction effects but these are not due to a therapeutic drug effect specific to the NAC group: acute treatment with the $DA_2$-R receptor antagonist haloperidol reduced the control response in the NAC and Naive groups and indeed more so in the latter (Besson et al., 1998).

### *3.4. Effects of pharmacological and/or psychotherapeutic treatment on helplessness in MDD patients*

*3.4.1. Study selection*

A systematic review was conducted of all randomised controlled human studies comparing measures of helplessness between patients receiving active treatment and those receiving either another active treatment or a control intervention or no intervention (e.g. wait-list control group). Appendix A Fig. A2 presents a flowchart of the literature search. Out of 166 human studies on the effects of pharmaco- or psycho-therapeutic treatment on measures of helplessness in MDD patients, identified by literature search and assessed for eligibility, 10 studies fulfilled the inclusion criteria and were included in quantitative analysis. In eight of these studies psychotherapy was the active intervention and in two of these studies combined psychotherapy–pharmacotherapy was the active intervention.

*3.4.2. Study characteristics*

Characteristics of the studies included are depicted in Table 5. Three studies focused on MDD (Lynch et al., 2003; Smit et al., 2005; Laidlaw et al., 2008), one study focused on major or minor depressive disorder or dysthymia (Singh et al., 2005), one study focused on remitted chronic MDD and dysthymia (Petersen et al., 2010), two studies focused on depression not further specified (Tsang et al., 2006; Liu, Chen, et al., 2009), and three studies focused on co-morbid depressive disorder in medical patients (Brody et al., 2006; Freedland et al., 2009; Sims et al., 2009). Four studies included patients identified with clinical interviews (Smit et al., 2005; Brody et al., 2006; Laidlaw et al., 2008; Freedland et al., 2009), three studies included patients with values above cut-off scores in established depression scales (Singh et al., 2005; Tsang et al., 2006; Liu, Chen, et al., 2009), and in three studies diagnosis by clinical interview plus above cut-off scores in established depression scales were required for inclusion (Lynch et al., 2003; Sims et al., 2009; Petersen et al., 2010).

Active psychotherapeutic approaches included depression recurrence prevention (Smit et al., 2005), self-management (Brody et al.,

**Table 4**
Overview of studies that assessed test-phase control behaviour in rodents after pre-exposure to uncontrollable aversive stimulation and pharmacological challenge.

| Reference | Inescapable variable | Dependent variable | | Description of injection | | | | Effect | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Apparatus | Measure | Drug | Acute/ chronic | Injection time before test | Concentration | Type of injection | Group | Drug | Group × drug |
| Hunziker, 1992 | Foot electro shock | 2-way shuttlebox | Locomotor transfer: escape | Naloxone (μ-opioid receptor antagonist) | Acute 1× | 10 min | 5/10/20 mg/kg | ip | Yes | Yes | Esc Lat reduced in IS; Esc Lat increased in NS |
| Telner & Singhal, 1981 | | | | Nortriptyline (tricyclic antidepressant) | Acute 1× | 30 min | 2/4/6/12.5 mg/kg | ip | Yes: Esc Resp + Esc Lat | No: Esc Resp + Esc Lat | Esc Lat and Esc Resp reduced in IS |
| Anisman et al., 1980[b] | | 2-way shuttlebox with CS | Locomotor transfer: avoid/escape | Apomorphine hydrochloride (dopamine receptor agonist) | Acute 1× | 15 min | 0.3/1.5/3 mg/kg | ip | Yes | No | Esc Lat reduced in IS |
| | | | | Clonidine (norepinephrine receptor agonist) | Acute 1× | 15 min | 0.75/1.5 mg/kg | ip | Yes | No | Esc Lat reduced in IS |
| Besson et al., 1998 | | | | Haloperidol (D2-dopamine receptor antagonist) | Rep. acute 3× | 15 min (all 3 times) | 37.5/75/150 mg/kg | ip | Yes | Yes | Esc Fail increased in NS>IS |
| | | | | Haloperidol and morphine (D2-dopamine receptor antagonist and μ-opioid receptor agonist) | Hal: as above Mor: sub chronic 5× | Hal: as above Mor: day 1: 2 h after stress (dose 1) days 2–5: bef. test (dose 2) after test: (dose 3) | Hal: as above; Mor: dose1: 6 mg/kg dose2: 4 mg/kg dose 3: 2 mg/kg | Hal: ip; Mor: sc | Yes | No: Morphine alone No: Morphine + haloperidol | Yes: Morphine alone reduced Esc Fail in IS no: morphine + haloperidol |
| Besson et al., 1996 | | | | Morphine (μ-opioid receptor agonist) | Sub chronic 5× | Mor: day 1: 1× days 2–5: 2× | 0.25/0.5/1/2/4/8 mg/kg | sc | Yes | No | Esc Fail reduced in IS |
| | | | | Naloxone (μ-opioid receptor antagonist) | Rep. acute 3× | 10 min | 0.25/0.5/1/2 mg/kg | ip | Yes | Yes: Esc Fail increased in NS and IS | No |
| | | | | Naloxone and morphine (μ-opioid receptor agonist) | Nal: rep. acute. 3× Mor: sub chronic 5× | As both above | Nal: 0.5 mg/kg; Mor: 1/8 mg/kg | Nal: ip Mor: sc | No NS | Yes: Nal antagonises Mor effect on Esc Fail in IS | |
| Porsolt et al., 1989 | | | | Hydroxyzine (antihistamine) | Sub. chronic 5× | 30 min (all 3 tests) | 8/16/32 mg/kg | ip | Yes | No | No |
| | | | | Diazepam (GABAa-receptor agonist) | Sub. chronic 5× | 30 min (all 3 tests) | 2 mg/kg | ip | Yes | No | No |
| Joca et al., 2006 | | | | Zimelidine (SSRI) | Acute 1× | After stress | 100 nmol/0.5 μl | Intracr. Dorsal hippocampus | Yes | No | Esc/avoid Lat reduced in IS |
| | | | | Desipramine (tricyclic antidepressant) | Acute 1× | After stress | 3/30 nmol/0.5 μl | Intracr. dorsal hippocampus | Yes | No | Esc/avoid Lat reduced in IS |
| | | | | Zimelidine (SSRI) | Acute 1× | 2 h after stress | 100 nmol/0.5 μl | Intracr. dorsal hippocampus | No | No | No |
| | | | | Desipramine (tricyclic antidepressant) | Acute 1× | 2 h after stress | 3/30 nmol/0.5 μl | Intracr. dorsal hippocampus | No | No | No |
| Danchev et al., 1989 | | | Locomotor transfer: avoid | Salbutamol (β2-adrenoceptor agonist) | Chronic 14× | 24 h (all 3 tests) | 1/5 mg/kg | Orally | No | No | No drug in NS |
| | | | | Trimethoquinol (β2-adrenoceptor agonist) | Chronic 14× | 24 h (all 3 tests) | 1/5 mg/kg | Orally | No | No | No drug in NS |

| Reference | Inescapable variable | Dependent variable | | | Description of injection | | | | Effect | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Apparatus | Measure | Drug | Acute/chronic | Injection time before test | Concentration | Type of injection | Group | Drug | Group × drug | |
| | | | | | Propanol (β2-adrenoceptor antagonist) | Chronic 14× | 24 h (all 3 tests) | 1/3 mg/kg | Orally | Yes | Yes | No drug in NS |
| | | | | | 3B (β2-adrenoceptor antagonist) | Chronic 14× | 24 h (all 3 tests) | 0.3/1/3 mg/kg | Orally | Yes | Yes | No drug in NS |
| Harrell et al., 1978 | | | Operant arena | Lever press = shock termination | Aramine (metaraminol bitartrate) (α1-adrenoceptor agonist) | Acute 1× | 20 min | 0.002 mg/kg | ?? | Yes | Yes | Esc Lat reduced in IS and NS |
| Sherman & Petty, 1980 | | | | | Desipramine (tricyclic antidepressant) | Acute 1× | 1 h | 1 μg | Intracr. FNC | Yes | No | Esc Fail reduced in IS |
| | | | | | Desipramine (tricyclic antidepressant) | Acute 1× | 1 h | 1 μg | Intracr. HC, SEPT, LGB[c] | Yes | No | No |
| | | | | | GABA | Acute 1× | 1 h | 1 μg | Intracr. HC, LGB | Yes | No | Esc Fail reduced in IS |
| | | | | | GABA | Acute 1× | 1 h | 1 μg | Intracr. FNC, SEPT[c] | Yes | No | No |
| | | | | | NE | Acute 1× | 1 h | 1 μg | Intracr. FNC, HC, SEPT, LGB[c] | Yes | No | No |
| | | | | | 5-HT | Acute 1× | 1 h | 1 μg | Intracr. FNC, SEPT | Yes | No | Esc Fail reduced in IS |
| | | | | | 5-HT | Acute 1× | 1 h | 1 μg | Intracr. HC, LGB[c] | Yes | No | No |
| Whitehouse et al., 1985[a] | | | | | Naltrexone opioid receptor antagonist | Acute 1× | 15 min | 10 mg/kg | ip | Yes: IS versus. ES/NS | No | Yes: Esc Lat reduced in IS; Esc Lat increased in ES/NS |
| Hemingway & Reigle, 1987[a] | | | | | Naltrexone (opioid receptor antagonist) | Acute 1× | 10 min | 3 mg/kg | No data | Yes: IS versus. ES/NS | No | No |
| Petty 1979 | | No data | Operant arena | Lever press = shock termination | Imipramine (tricyclic antidepressant) | Sub chronic 5× | 1 h | 1/2/5/10/20/40 mg/kg | ip | Yes | Yes: high subchronic dose | No drug in NS |
| Grahn et al., 2002 | | Tail electro shock | 2-way shuttle box | Locomotor transfer: escape | Benoxathian (α1-adrenoceptor antagonist) | Acute 1× | Bef. test | 4 μg/1 μl | Intracr. DRN | Freez: yes Esc Lat: yes | Freez: no Esc Lat: no | Freezing reduced in IS Esc Lat: no |
| Grahn et al., 2000 | | | | | APV (2-amino-5-phospho-novaleric acid) (NMDA-receptor antagonist) | acute 1x | 10 min | 5 ng/1 μl | Intracr. DRN | Freez: yes Esc Lat: yes | Freez: no Esc Lat: no | Freezing reduced in IS Esc Lat increased in NS |
| | | | | | L-NAME (L-N-nitro-L-arginine methyl ester) (Nitric oxide synthase inhibitor) | Acute 1× | 10 min | 5 μg/1 μl | Intracr. DRN | Freez: yes Esc Lat: yes | Freez: no Esc Lat: no | Freezing reduced in IS Esc Lat reduced in IS |

SSRI, selective serotonin reuptake inhibitor; 5-HT, serotonin; GABA, gamma-aminobutyric acid; NE, noradrenaline; Rep. Acute, repeated acute schedule; ip,intra-peritoneal; sc, sub-cutaneous; intracr., intra-cranial; DRN, dorsal raphé nucleus; FNC, frontal neocortex; HC, hippocampus; LGB, lateral geniculate body; SEPT, septum
ES, escapable stressor; IS, inescapable stressor; NS, no stressor; Esc Fail, escape failure; Esc Lat, escape latency; Freez, Freezing response

[a] These studies were conducted using a triadic design.
[b] These studies were conducted in mice.
[c] Additional injection sites that were studied: entorhinal cortex, posterior neocortex, caudate, n. accumbens and amygdala.

**Table 5**
Overview of RCT studies that assessed measures of helplessness in MDD patients before and after treatment.

| Reference | Subjects | | Independent variable | | Dependent variable | | Sample size | Major results | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Diagnosis of patient | Diagnostic instrument of depression (interview/ questionnaire) | Treatment intervention | Control intervention | Target construct | Scale | | Changes from pre- to post-treatment | Group differences post-intervention | Delta pre-post-differences between groups |
| Smit et al., 2005 | Major depressive disorder (DSM-IV) | Composite International Diagnostic Interview | Depression-Recurrence Prevention (DRP); DRP + Psychiatrist (DRPp); DRP + Cognitive Behaviour Therapy (DRPcbt) | Care as usual (CAU) | Perceived self-efficacy for managing depression | Depression Self-Efficacy Scale | 267 | DRP: pre < post CAU: pre < post DRPp: pre = post DRPcbt: pre = post | DRP = DRPp = DRPcbt = CAU | ns |
| Brody et al., 2006 | Major or minor depressive disorder (DSM-IV) in patients with age-related macular degeneration | Structured Clinical Interview for DSM-IV | 12-hour self-management program (SM) | 12-hour tape-recorded health education; Wait list | Expectations for handling defined situations related to macular degeneration | Macular Degeneration Self-Efficacy Scale | 32 | SM: pre < post Controls: pre = post Further results: SM: decrease of depressive symptoms associated with increase in self-efficacy (not associated in control groups); coefficient of association significantly different between groups | SM = controls | SM > controls |
| Freedland et al., 2009 | Major or minor depressive disorder (DSM-IV) in patients who had undergone coronary artery bypass graft surgery in the past year | Depression Interview and Structured Hamilton | Cognitive Behaviour Therapy (CBT); Supportive Stress Management (SM) | Care as usual (CAU) | Hopelessness towards the future | Beck Hopelessness Scale | 123 | Total group: pre < post | CBT = SM; CBT > CAU; SM = CAU | ns |
| Laidlaw et al., 2008 | Major depressive disorder (DSM-IV) | Schedule for Affective Disorders and schizophrenia — Life time version | Cognitive Behaviour Therapy (CBT) | Care as usual (CAU) | Hopelessness towards the future | Beck Hopelessness Scale | 44 | CBT: pre < post CAU: pre = post | CBT = CAU | CBT > CAU at 6 months only |

| Reference | Subjects | | Independent variable | | Dependent variable | | Sample size | Major results | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Diagnosis of patient | Diagnostic instrument of depression (interview/ questionnaire) | Treatment intervention | Control intervention | Target construct | Scale | | Changes from pre- to post-treatment | Group differences post-intervention | Delta pre-post-differences between groups |
| Lynch et al., 2003 | Major depressive disorder (DSM-IV) | Duke Depression Evaluation Schedule and (Hamilton Rating Scale for Depression-17, cut-off: >=19 or Beck Depression Inventory, cut-off: >=19) | Dialectical Behaviour Therapy + Medication (DBT + MED) | Medication only (MED) | Hopelessness towards the future | Beck Hopelessness Scale | 34 | DBT + MED: pre<post; MED: pre=post | DBT + MED = MED | ns |
| Singh et al., 2005 | (Major or minor depressive disorder or dysthymia (DSM-IV)) | Geriatric Depression Scale, cut-off: >=14 | High intensity progressive resistance training (HPRT) | Low intensity progressive resistance training (LPRT); Care as usual (CAU) | Health locus of control (LC); Self-efficacy (SE) regarding behaviour | Self-Efficacy Scale; Multidimensional Health Locus of Control | 60 | Total group: LC: pre=post; SE: pre<post | LC: not reported; SE: HPRT=LPRT; SE: HPRT>CAU | LC: ns; SE: ns |
| Sims et al., 2009 | "Depression" (not further specified) in stroke survivors | Patient Health Questionnaire-9; Present State Examination, depression module | Progressive resistance training (PRT) | Wait list (WL) | Recovery locus of control | Recovery Locus of Control Scale | 45 | PRT: pre<post; WL: not reported | Not reported | Not reported |
| Liu, Chen, et al., 2009 | Depression | Beck Depression Inventory-II, cut-off: 10<patient<47 and not suicidal ideation | Cognitive Bibliotherapy (CB) | Wait list (WL) | Learned resourcefulness | Self-Control Schedule | 52 | CB: pre<post; WL: pre=post<br>Learned resourcefulness partially mediated the effect of CB on cognitive-affective symptoms of depression. | CB>WL | CB>WL |
| Petersen et al., 2010 | Remitted chronic major depressive disorder (and dysthymia) (DSM-III-R) | Acute phase: Structured Clinical Interview for DSM-III-R; Hamilton Rating Scale for Depression>=16; Remission: Hamilton Rating Scale for Depression<=7 | Cognitive Behaviour Therapy + placebo (CBTp); Fluoxetine (40 mg) only (F); Cognitive Behaviour Therapy + fluoxetine (40 mg) (CBTf) | Placebo only (P) | Hopelessness towards the future | Beck Hopelessness Scale | 55 | Inference statistics not reported<br>Descriptive: CBTp: pre<post; CBTf: pre<post; F: pre>post; P: pre>post | Not reported | ns |
| Tsang et al., 2006 | Depression (not further specified: diagnosis or obvious features) | Geriatric Depression Scale, cut-off: total score >8 | Qigong (Q) | Newspaper reading group (N) | Confidence in ability to deal with novel or demanding situations | The Chinese Self-Efficacy Scale | 82 | Q: pre<post; N: pre>post | Q>N | Q>N |

Note: < worse than, > better than, ns not significant

2006), cognitive behaviour therapy (Laidlaw et al., 2008; Freedland et al., 2009; Petersen et al., 2010), dialectical behaviour therapy (Lynch et al., 2003), progressive resistance training (Singh et al., 2005; Sims et al., 2009), cognitive bibliotherapy (Liu, Chen, et al., 2009) and qigong (Tsang et al., 2006). Active pharmacotherapeutic approaches included fluoxetine (Petersen et al., 2010) or standard antidepressant medication not further specified (Lynch et al., 2003). One study compared its active treatment with another active treatment (Tsang et al., 2006), three studies compared active treatments with care-as-usual (Smit et al., 2005; Laidlaw et al., 2008; Freedland et al., 2009), two studies compared active treatments with wait list (Liu, Chen, et al., 2009; Sims et al., 2009), two studies compared their active treatments with both another active treatment and wait list or care as usual (Singh et al., 2005; Brody et al., 2006), two studies compared active treatments with placebo or medication (Lynch et al., 2003; Petersen et al., 2010).

Helplessness-related target outcomes of interest included self-efficacy (Singh et al., 2005; Smit et al., 2005; Brody et al., 2006; Tsang et al., 2006), hopelessness (Lynch et al., 2003; Laidlaw et al., 2008; Freedland et al., 2009; Petersen et al., 2010), locus of control (Singh et al., 2005; Sims et al., 2009), and learned resourcefulness (Liu, Chen, et al., 2009).

*3.4.3. Results of individual studies and synthesis of results*

Major study findings are presented in Table 5.

*Changes from pre-to post-treatment*: in all studies, measures of helplessness were improved by the active psychosocial treatment, except for Smit et al. (2005) in which the depression-recurrence prevention in combination with either psychiatrist-consulting or cognitive behaviour therapy did not improve self-efficacy. In the Singh et al. (2005) study, health locus of control did not change from pre- to post-intervention. Pharmacological treatment alone either resulted in no change or in deterioration of helplessness measures (Lynch et al., 2003; Petersen et al., 2010).

*Treatment group differences post-treatment*: in four studies, patients in the active treatment group showed less helplessness post-treatment as compared to the patient control group. The active treatments were cognitive behaviour therapy, high-intensity progressive resistance training, cognitive bibliotherapy, or qigong (Singh et al., 2005; Tsang et al., 2006; Freedland et al., 2009; Liu, Chen, et al., 2009). With regard to the type of helplessness measure, the reduction was seen for self-efficacy (Singh et al., 2005; Tsang et al., 2006), hopelessness (Freedland et al., 2009), and learned resourcefulness (Liu, Chen et al., 2009). No differences between active treatment and control groups at post-treatment were seen for: self-efficacy, using the depression-recurrence prevention programme (Smit et al., 2005), the self-management programme (Brody et al., 2006), or high-intensity resistance training (Singh et al., 2005); hopelessness, using one cognitive behaviour therapy trial (Laidlaw et al., 2008), supportive stress management (Freedland et al., 2009), or dialectical behavioural therapy plus medication (Lynch et al., 2003). Two studies, focusing on locus of control and hopelessness, did not report data regarding post-treatment group differences (Sims et al., 2009; Petersen et al., 2010).

*Delta of the pre-post differences between treatment groups*: in four studies, the active treatment, including self-management, cognitive behaviour therapy, cognitive bibliotherapy, and qigong, resulted in greater improvement of self-efficacy, hopelessness or learned resourcefulness, than did the control interventions (Brody et al., 2006; Tsang et al., 2006; Laidlaw et al., 2008; Liu, Chen, et al., 2009). This was not seen for: depression-recurrence prevention programme focusing on self-efficacy (Smit et al., 2005); cognitive behaviour therapy, supportive stress management, fluoxetine, or dialectical behavioural therapy plus medication focusing on hopelessness (Lynch et al., 2003; Freedland et al., 2009; Petersen et al., 2010), or high-intensity progressive resistance training focusing on self-efficacy and locus of control (Singh et al., 2005). One study, focusing on locus of control, did not report data regarding the group differences of changes from pre-to post-treatment (Sims et al., 2009).

Therefore, focusing on pharmacotherapy and helplessness in MDD, there is currently no RCT study for depression that has included a measure of helplessness as target outcome and has demonstrated a therapeutic effect for an antidepressant drug in terms of an improvement on the helplessness measure. According to the search, one RCT study has been conducted with helplessness as a target outcome and with a pharmacotherapy-only arm. This was a study with the anti-depressant fluoxetine: scores on the BHS were increased after fluoxetine treatment whereas cognitive behaviour therapy reduced BHS scores in the same study.

## 4. Discussion

Firstly, a review and integration of the theory and evidence for the learned helplessness effect in rodents, and that for the learned helplessness effect, the learned helplessness theory of depression and helplessness in depression, in humans, are presented. The LH effect is a relatively specific psychological state that translates across species. For rodents, primarily the laboratory rat, there is evidence that emotional, motivational and cognitive effects of uncontrollable aversive stimulation mediate the LH effect. In humans, the LH effect and the LH theory of depression have been largely treated as cognitive processes. At the neurobiological level, a circuitry model of the LH effect in the rat proposes that both controllable and uncontrollable aversive stimuli activate CNS punishment systems, such as serotonergic output from the DRN in response to painful stimuli. The controllability of the stimulus is proposed to be processed in the vmPFC, the rat analogue of the human ACC. The presence of control is relayed out of the vmPFC to regions sensitive to aversive stimulation per se, to reduce the response of these punishment systems, such that a state of controllability is maintained/LH effect is prevented. Human imaging and EEG findings provide evidence that perceived state of uncontrollability is associated positively with ACC activity, and in MDD patients perceived state of helplessness is associated positively with ACC/PFC activity. Secondly, systematic reviews are presented for studies of the effects of pharmacological agents on control behaviour in rodents exposed to uncontrollable aversive stimulation, and of pharmaco- and/or psycho-therapy in MDD patients on measures and reports of helplessness and related constructs.

As discussed below, the findings of these reviews and systematic reviews allow for clarification of a number of translational issues and serve to identify areas for future translational research into the LH effect and helplessness. It would appear that there is real potential for translational research into the LH effect and helplessness to yield important novel insights into the neuropsychopathology of depression, in terms of aetiology, maintenance and treatment.

### 4.1. Clarification of translational issues

*4.1.1. Uncontrollability versus helplessness*

The use of the term helplessness to describe the robust but relatively situation-specific, short-term effect of uncontrollable aversive stimuli on behaviour that is the LH effect, would appear to have inhibited progress, within and between animal and human studies, in our understanding of helplessness and its relevance to MDD aetiology, onset and maintenance. Within animal studies, the term LH as applied to the AC-NAC-Naive model has contributed to the general view that this and other, situation-specific short-term manipulations, such as the forced swim test and tail suspension test, induce helplessness and constitute models of helplessness in depression (Pryce & Seifritz, 2011). Within human studies, the term LH has also been associated with a too literal extrapolation of the effects of specific uncontrollable aversive stimuli on behaviour of healthy individuals to the psychopathological state of helplessness in depression. To use a more parsimonious terminology, what the LH effect demonstrates is learned aversive uncontrollability (LAU). The LAU effect has been demonstrated in healthy animals and healthy humans. The LAU

effect demonstrates the sensitivity of healthy individuals to the unique organism–environment situation of uncontrollability.

An intense, chronic LAU effect, elicited by a major aversive stimulus/life event that is uncontrollable, could well be aetiological in inducing generalised helplessness, which in turn could be the major aetiological and maintenance factor in MDD, and the necessary focus of its treatment. Re-naming the LH effect as the LAU effect is aimed at clarifying and not de-valuing the importance of this effect. On the contrary, by using the more parsimonious descriptor of the LAU effect, the inter-relationships between the LAU effect and generalised helplessness and between generalised helplessness and MDD can be clarified. This clarification should serve to facilitate research aimed at furthering understanding of these inter-relationships, within and between animals and humans.

#### 4.1.2. Cognitive versus emotional–motivational–cognitive causality

The major strength of the LH effect (hereafter referred to as the LAU effect) is the non-ambiguity of the independent variable, namely aversive uncontrollability. This clarity results in a marked aetiological and construct validity of the LAU effect relative to other aversive stimulation models used in preclinical depression research (Pryce & Seifritz, 2011). The psychological processes proposed to underlie the effect, namely a constellation of emotional, motivational and cognitive processes, and their interactions, would appear to represent a realistic assessment of the complexity of the LAU effect. Furthermore, studies of each of these processes and the changes in their state as a consequence of uncontrollability, is likely to be necessary for a proximate understanding of the LAU effect. These same processes and their interactions are also major candidates as aetiological and maintenance factors in the psychopathology of helplessness in MDD. It is unfortunate, therefore, that the discussion of the relevance of LH to MDD, including the LH theory of MDD, has focused on cognitive processes, such as contingency and attribution, to the near exclusion of the role of emotional and motivational processes. Psychological theories such as incentive-motivation, which integrate physiological motivation with cognitive processes such as goal-directed expectancy (e.g. Balleine & Dickinson, 1998), are clearly relevant and need to be integrated into theoretical and empirical helplessness research in the future.

#### 4.1.3. Neurobiological translation

Animal and human studies of the neurobiological underpinnings of the LAU effect and helplessness are still at an early stage but already some striking parallels have been identified, primarily in terms of a central role for analogous PFC regions and an important modulatory role of 5-HT in these regions. These findings suggest that the mPFC/ACC is central to both the LAU effect and the state of helplessness in MDD, and could therefore be important in mediating any causal relationship between these two processes.

### 4.2. Directions for future research

#### 4.2.1. From the learned aversive uncontrollability effect back to the learned helplessness effect

If it is accepted to be constructive to re-define the LH effect as the LAU effect, then this should facilitate the design of rodent models that do indeed measure an LH effect i.e. that demonstrate a generalised chronic state of a deficit in responding with control or expectancy in contexts and towards stimuli other than those used to induce this state. In LAU models it is per definition established that a deficit in a specific control response in the NAC group is the result of experiencing aversive uncontrollability. This deficit can then serve as a comparator, such that should animals exhibit the same deficit following exposure to other aversive environments, this deficit can be interpreted as being underlain by the psychological state of generalised uncontrollability i.e. a true LH effect. Candidate aversive manipulations are chronic unpredictable stress or chronic social defeat stress (e.g. Krishnan & Nestler, 2008). Furthermore, in such models the control deficit used to define the LH state is likely to co-occur with a number of other depression-relevant state markers e.g. loss of body weight and condition, disturbance of circadian activity, reduced wanting and liking of rewarding stimuli, increased fatigability. These models will allow for the study of the inter-relationships between LH and other state markers, including whether treatments that increase control/reduce LH also lead to reduction of the other markers.

#### 4.2.2. The importance of individual differences

A characteristic of the original LH effect model in rodents and humans is that subjects are allocated randomly to the study groups i.e. AC, NAC, Naive. The underlying assumption is that the average sensitivity to uncontrollability will be equal between the individuals in the yoked AC and NAC groups. Assessment of the (un)controllability of an aversive environment is of course individual-specific and these individual differences will be extremely important. Depending on the paradigm, a proportion of individuals allocated to the AC group would be expected to experience the controllable aversive stimulus/context as (to some extent) uncontrollable and to develop a LAU state. Also, a proportion of individuals allocated to the NAC group would be expected to persist with control attempts and to be resistant to developing a LAU state. This variation is to be welcomed, given that LAU effect and LH effect models need to provide insights into the human inter-individual differences in vulnerability-resilience in responses to aversive uncontrollability. Animal models of the LAU effect and generalised LH effect that are designed to allow for the study of the contribution of (epi-)genetic and development–environmental factors to these effects will be of high validity and importance.

#### 4.2.3. Translational models and studies based on dynamic psychological–neurobiological processes

According to current LAU effect theory based on the rat data, aversive stimuli exert acute effects on the brain in terms of stimulating emotional, motivational and cognitive processing. Any perceived uncontrollability of these aversive stimuli will feed back onto these psychological processes and therefore onto the neurobiological processes underlying them. Extrapolating to the human situation, in a situation where a life event of high emotional significance is being proposed to lead to a chronic state of helplessness because of its uncontrollability, then this situation also needs to be studied at the reciprocal level of neurobiological processes controlling psychological responses and psychological state feeding back onto neurobiological state. To-date, the emphasis has been more on the acute onset of the LAU effect and less on how this effect can lead to chronic, bi-directional feedback between psycho(patho)logical and neuro(patho)logical states.

#### 4.2.4. Valid models and state markers for drug discovery and development

The primary rationale for improved understanding of the LH effect and of helplessness as they relate to depression is to facilitate the accurate assessment of the importance and relevance of helplessness as a psychopathological target for therapy, and the discovery and development of effective pharmacological and psychotherapeutic treatments with which to achieve this. It is hoped that the current review will make a helpful contribution here, and will stimulate much-needed advancement in our understanding and treatment of MDD.

## Acknowledgments

This research was supported by The Swiss National Science Foundation: grant 31003A_130499 and the National Center for Competence in Research “Neural Plasticity and Repair”.

## Appendix A

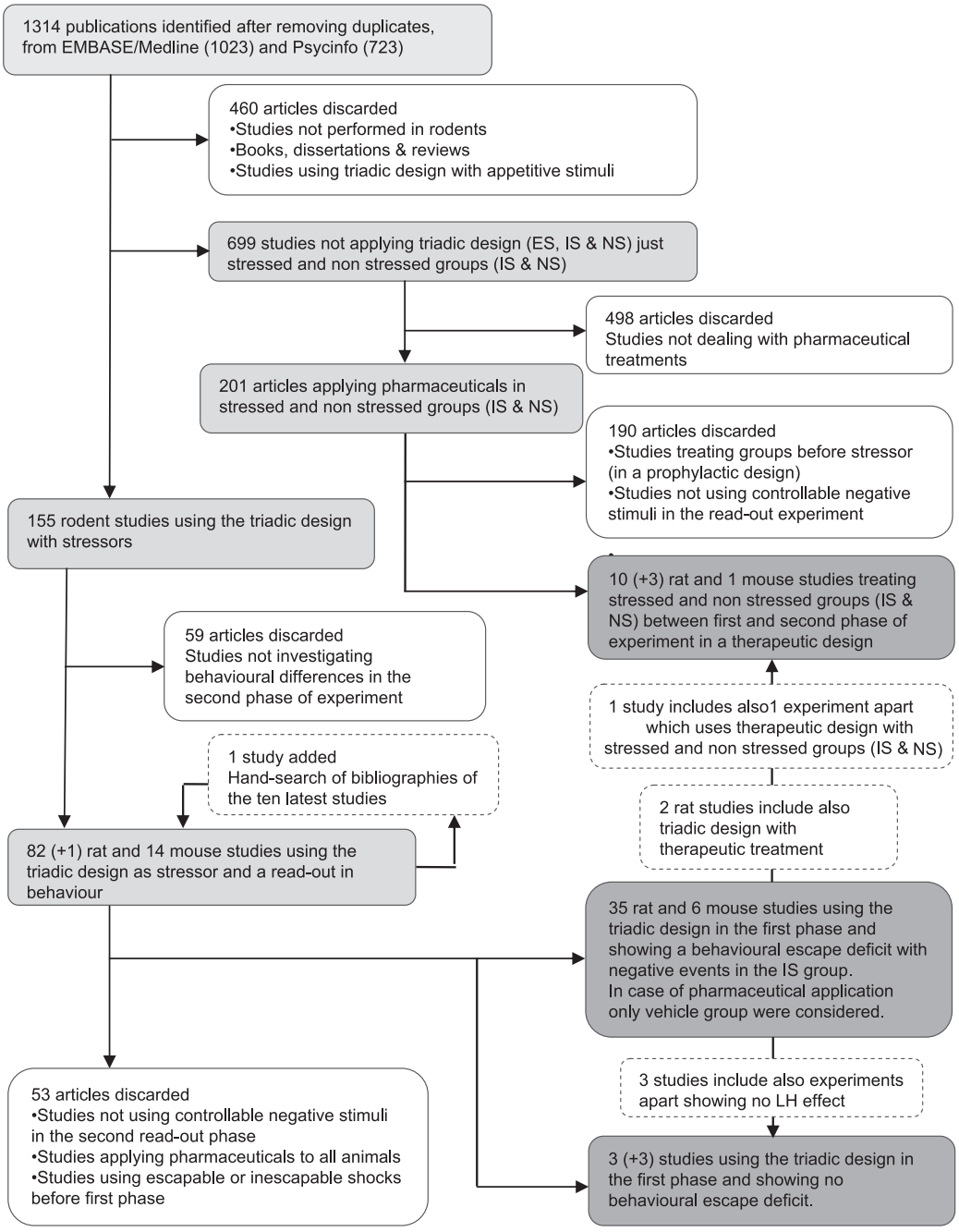



**Fig. A1.** Flowchart of the literature search for studies on the learned helplessness effect and on test-phase control behaviour in rodents after pre-exposure to uncontrollable aversive stimulation and pharmacological challenge.

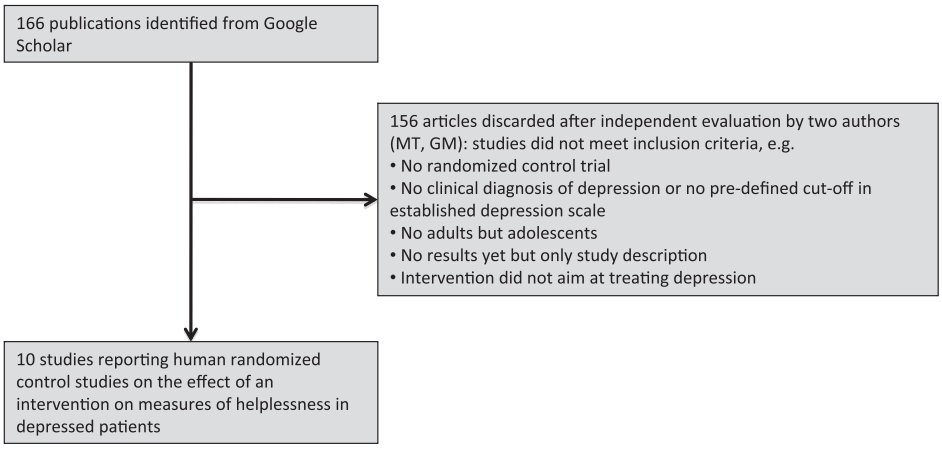



**Fig. A2.** Flowchart of the literature search for randomised control studies on measures of helplessness and controllability in MDD patients and control probands after therapeutic treatment.

## References


Abramson, L. Y., Metalsky, G. I., & Alloy, L. B. (1989). Hopelessness depression: a theory-based subtype of depression. *Psychol Rev 96*, 358–372.

Abramson, L. Y., Seligman, M. E., & Teasdale, J. D. (1978). Learned helplessness in humans: critique and reformulation. *J Abnorm Psychol 87*, 49–74.

Agid, O., Kohn, Y., & Lerer, B. (2000). Environmental stress and psychiatric illness. *Biomed Pharmacother 54*, 135–141.

Altenor, A., Kay, E., & Richter, M. (1977). The generality of learned helplessness in the rat. *Learn. Motiv 8*, 54–61.

Amat, J., Baratta, M., Paul, E., Bland, S., Watkins, L., & Maier, S. (2005). Medial prefrontal cortex determines how stressor controllability affects behavior and dorsal raphe nucleus. *Nat Neurosci 8*, 365–371.

Amat, J., Paul, E., Watkins, L. R., & Maier, S. F. (2008). Activation of the ventral medial prefrontal cortex during an uncontrollable stressor reproduces both the immediate and long-term protective effects of behavioral control. *Neuroscience 154*, 1178–1186.

Anisman, H., deCatanzaro, D., & Remington, G. (1978). Escape performance following exposure to inescapable shock: deficits in motor response maintenance. *J Exp Psychol Anim Behav Process 4*, 197–218.

Anisman, H., DeCatanzaro, D., & Remington, G. (1978). Escape performance following exposure to inescapable shock: deficits in motor response maintenance. *J Exp Psychol Anim Behav Process 4*, 197–218.

Anisman, H., Hamilton, M., & Zacharko, R. M. (1984). Cue and response-choice acquisition and reversal after exposure to uncontrollable shock: induction of response perseveration. *J Exp Psychol Anim Behav Process 10*, 229–243.

Anisman, H., Irwin, J., & Sklar, L. S. (1979). Deficits of escape performance following catecholamine depletion: implications for behavioral deficits induced by uncontrollable stress. *Psychopharmacology 64*, 163–170.

Anisman, H., & Merali, Z. (2001). Rodent models of depression: learned helplessness induced in mice. *Curr Prot Neurosci Behav Neurosci Suppl 14* 810C.811-810C.815.

Anisman, H., Pizzino, A., & Sklar, L. S. (1980). Coping with stress, norepinephrine depletion and escape performance. *Brain Res 191*, 583–588.

Anisman, H., Suissa, A., & Sklar, L. S. (1980). Escape deficits induced by uncontrollable stress: antagonism by dopamine and norepinephrine agonists. *Behav Neural Biol 28*, 34–47.

Ansorge, M. S., Hen, R., & Gingrich, J. A. (2007). Neurodevelopmental origins of depressive disorders. *Curr Opin Pharmacol 7*, 8–17.

Arnsten, A. F. T. (2009). Stress signalling pathways that impair prefrontal cortex structure and function. *Nat Rev Neurosci 10*, 410–422.

Balleine, B. W., & Dickinson, A. (1998). Goal-directed instrumental action: contingency and incentive learning and their critical substrates. *Neuropharmacol 37*, 407–419.

Baratta, M. V., Christianson, J. P., Gomez, D. M., Zarza, C. M., Amat, J., Masini, C. V., et al. (2007). Controllable versus uncontrollable stressors bi-directionally modulate conditioned but not innate fear. *Neuroscience 146*, 1495–1503.

Bauer, H., Pripfl, J., Lamm, C., Prainsack, C., & Taylor, N. (2003). Functional neuroanatomy of learned helplessness. *Neuroimage 20*, 927–939.

Beck, A. T., & Steer, R. A. (1988). *Manual for the Beck Hopelessness Scale.* Tex: San Antonia.

Beck, A. T., Ward, C. H., Mendelson, M., Mock, J., & Erbaugh, J. (1961). An inventory for measuring depression. *Arch Gen Psychiatry 4*, 561–571.

Belmaker, R. H., & Agam, G. (2008). Major depressive disorder. *N Engl J Med 358*, 55–68.

Berridge, K. C., & Robinson, T. E. (2003). Parsing reward. *TINS 26*, 507–513.

Bersh, P. J., Whitehouse, W. G., Blustein, J. E., & Alloy, L. B. (1986). Interaction of Pavlovian conditioning with a zero operant contingency: chronic exposure to signaled inescapable shock maintains learned helplessness effects. *J Exp Psychol Anim Behav Process 12*, 277–290.

Bersh, P. J., Whitehouse, W. G., Laurence, M. T., Blustein, J. E., & Alloy, L. B. (1990). Signaling the duration of uncontrollable shock impairs subsequent shock escape. *Psychol Rec 40*, 113–125.

Besson, A., Privat, A. M., Eschalier, A., & Fialip, J. (1996). Effects of morphine, naloxone and their interaction in the learned-helplessness paradigm in rats. *Psychopharmacology 123*, 71–78.

Besson, A., Privat, A., Eschalier, A., & Fialip, J. (1998). Reversal of learned helplessness by morphine in rats: involvement of a dopamine mediation. *Pharmacol Biochem Behav 60*, 519–525.

Bhagwager, Z., Hinz, R., Taylor, M., Fancy, S., Cowen, P., & Grasby, P. (2006). Increased 5-HT(2A) receptor binding in euthymic, medication-free patients recovered from depression: a positron emission study with [(11)C]MDL 100,907. *Am J Psychiatry 163*, 1580–1587.

Blettner, M., Sauerbrei, W., Schlehofer, B., Scheuchenpflug, T., & Friedenreich, C. (1999). Traditional reviews, meta-analyses and pooled analyses in epidemiology. *Int J Epidemiol 28*, 1–9.

Bolz, J., & Giedke, H. (1981). Controllability of an aversive stimulus in depressed patients and healthy controls: a study using slow brain potentials. *Biol Psychiatry 16*, 441–452.

Braud, W., Wepman, B., & Russo, D. (1969). Task and species generality of the "helplessness" phenomenon. *Psychon Sci 16*, 154–155.

Brody, B. L., Roch-Levecq, A. C., Kaplan, R. M., Moutier, C. Y., & Brown, S. I. (2006). Age-related macular degeneration: self-management and reduction of depressive symptoms in a randomized, controlled study. *J Am Geriatr Soc 54*, 1557–1562.

Brown, V. J., & Bowman, E. M. (2002). Rodent models of prefrontal cortical function. *TINS 25*, 340–343.

Brown, G. W., Harris, T. O., & Hepworth, C. (1995). Loss, humiliation and entrapment among women developing depression: a patient and non-patient comparison. *Psychol Med 25*, 7–21.

Brown, P., Hurley, C., Repucci, N., & Drugan, R. C. (2001). Behavioral analysis of stress controllability effects in a new swim stress paradigm. *Pharmacol Biochem Behav 68*, 263–272.

Buchwald, A. M., Coyne, J. C., & Cole, C. S. (1978). A critical evaluation of the learned helplessness model of depression. *J Abnorm Psychol 87*, 180–193.

Byers, A. L., Yaffe, K., Covinsky, K. E., Friedman, M. B., & Bruce, M. L. (2010). High occurrence of mood and anxiety disorders among older adults: the National Comorbidity Survey Replication. *Arch Gen Psychiatry 67*, 489–496.

Caspi, A., & Moffitt, T. E. (2006). Gene-environment interactions in psychiatry: joining forces with neuroscience. *Nat Rev Neurosci 7*, 583–590.

Caspy, T., Frommer, R., Weiner, I., & Lubow, R. (1979). Generality of US preexposure effects: effect of shock or food preexposure on water escape. *Bull Psychon Soc 14*, 15–18.

Caspy, T., & Lubow, R. E. (1981). Performance decrement following shock preexposure: the effect of number and duration of escapable and inescapable shocks. *Psychol Rec 31*, 183–194.

Celada, P., Puig, M. V., Casanovas, J. M., Guillazo, G., & Artigas, F. (2001). Control of dorsal raphe serotonergic neurons by the medial prefrontal cortex: involvement of serotonin-1A, GABA(A), and glutamate receptors. *J Neurosci 21*, 9917–9929.

Chiu, P. H., & Deldin, P. J. (2007). Neural evidence for enhanced error detection in major depressive disorder. *Am J Psychiatry 164*, 608–616.

Choudary, P. V., Molnar, M., Evans, S. J., Tomita, H., Li, J. Z., Vawter, M. P., et al. (2005). Altered cortical glutamatergic and GABAergic signal transmission with glial involvement in depression. *PNAS 102*, 15653–15658.

Chourbaji, S., Zacher, C., Sanchis-Segura, C., Dormann, C., Vollmayr, B., & Gass, P. (2005). Learned helplessness: validity and reliability of depressive-like states in mice. *Brain Res Protoc 16*, 70–78.

Cools, R., Roberts, A. C., & Robbins, T. W. (2007). Serotonergic regulation of emotional and behavioural control processes. *Trends Cogn Sci 12*, 31–40.
Cotton, M., & Smith, G. (1990). Prenatal stress and learned helplessness. *Aust J Psychol 42*, 47–55.
Cotton, M., et al. (1982). Learned helplessness in shuttlebox-avoidance behavior. *Psychol Rep 51*, 215–221.
Danchev, N., Staneva-Stoytcheva, D., & Loukova, S. (1989). Effect of beta-adrenergic and serotoninergic agents on the stress-induced behaviour of 'learned helplessness' in rats. *Acta Physiol Pharmacol Bulg 15*, 17–24.
Diener, C., Kuehner, C., Brusniak, W., Struve, M., & Flor, H. (2009). Effects of stressor controllability on psychophysiological, cognitive and behavioural responses in patients with major depression and dysthymia. *Psychol Med 39*, 77–86.
Diener, C., Kuehner, C., & Flor, H. (2010). Loss of control during instrumental learning: a source localization study. *Neuroimage 50*, 717–726.
Drugan, R. C., Eren, S., Hazi, A., Silva, J., Christianson, J. P., & Kent, S. (2005). Impact of water temperature and stressor controllability on swim stress-induced changes in body temperature, serum corticosterone, and immobility in rats. *Pharmacol Biochem Behav 82*, 397–403.
DSM-IV (1994). *Diagnostic and Statistical Manual of Mental Disorders. 4th Edn. American Psychiatric Association, Washington, DC*. Washington, DC: American Psychiatric Association.
Duman, R. S., Heninger, G., & Nestler, E. (1997). A molecular and cellular theory of depression. *Arch Gen Psychiatry 54*, 597–606.
Elliott, R., Rubinsztein, J. S., Sahakian, B. J., & Dolan, R. J. (2002). The neural basis of mood-congruent processing biases in depression. *Arch Gen Psychiatry 59*, 597–604.
Fawcett, J., Scheftner, W. A., Fogg, L., Clark, D. C., Young, M. A., Hedeker, D., et al. (1990). Time-related predictors of suicide in major affective disorder. *Am J Psychiatry 147*, 1189–1194.
Freedland, K. E., Skala, J. A., Carney, R. M., Rubin, E. H., Lustman, P. J., Davila-Roman, V. G., et al. (2009). Treatment of depression after coronary artery bypass surgery: a randomized controlled trial. *Arch Gen Psychiatry 66*, 387–396.
Fretska, E., Bauer, H., Loedolter, M., & Loedolter, U. (1999). Loss of control and negative emotions: a cortical slow potential topography study. *Int J Psychophysiol 33*, 127–141.
Gabbott, P. L., Warner, T. A., Jays, P. R., Salway, P., & Busby, S. J. (2005). Prefrontal cortex in the rat: projections to subcortical autonomic, motor, and limbic centers. *J Comp Neurol 492*, 145–177.
Gelenberg, A. J., Freeman, M. P., Markowitz, J. C., Rosenbaum, J. F., Thase, M. E., Trivedi, M. H., et al. (2010). *Practice Guideline for the Treatment of Patients with Major Depressive Disorder* (3rd ed.). Arlington, VA: American Psychiatric Publishing, Inc.
Gonzalez, H. M., Vega, W. A., Williams, D. R., Tarraf, W., West, B. T., & Neighbors, H. W. (2010). Depression care in the United States: too little for too few. *Arch Gen Psychiatry 67*, 37–46.
Grahn, R. E., Hammack, S., Will, M., O'Connor, K., Deak, T., Sparks, P., et al. (2002). Blockade of alpha1 adrenoreceptors in the dorsal raphe nucleus prevents enhanced conditioned fear and impaired escape performance following uncontrollable stressor exposure in rats. *Behav Brain Res 134*, 387–392.
Grahn, R. E., Kalman, B. A., Brennan, F. X., Watkins, L. R., et al. (1995). The elevated plus-maze is not sensitive to the effect of stressor controllability in rats. *Pharmacol Biochem Behav 52*, 565–570.
Grahn, R. E., Watkins, L. R., & Maier, S. F. (2000). Impaired escape performance and enhanced conditioned fear in rats following exposure to an uncontrollable stressor are mediated by glutamate and nitric oxide in the dorsal raphe nucleus. *Behav Brain Res 112*, 33–41.
Green, J. G., McLaughlin, K. A., Berglund, P. A., Gruber, M. J., Sampson, N. A., Zaslavsky, A. M., et al. (2010). Childhood adversities and adult psychiatric disorders in the national comorbidity survey replication I: associations with first onset of DSM-IV disorders. *Arch Gen Psychiatry 67*, 113–123.
Grimm, S., Boesiger, P., Beck, J., Schuepbach, D., Bermpohl, F., Walter, M., et al. (2009). Altered negative BOLD responses in the default-mode network during emotion processing in depressed subjects. *Neuropsychopharmacol 34*, 932–943.
Grimm, S., Ernst, J., Boesiger, P., Schuepbach, D., Hell, D., Boeker, H., et al. (2009). Increased self-focus in major depressive disorder is related to neural abnormalities in subcortical–cortical midline structures. *Hum Brain Mapp 30*, 2617–2627.
Haber, S. N., & Knutson, B. (2010). The reward circuit: linking primate anatomy and human imaging. *Neuropsychopharmacol 35*, 4–26.
Hamilton, M. (1960). A rating scale for depression. *J Neurol Neurosurg Psychiatry 23*, 56–62.
Hamzaoglu, O., Ozkan, O., Ulusoy, M., & Gokdogan, F. (2010). The prevalence of hopelessness among adults: disability and other related factors. *Int J Psychiatry Med 40*, 77–91.
Harrell, E. H., Haynes, J. R., Lambert, P. L., & Sininger, R. A. (1978). Reversal of learned helplessness by peripheral arousal. *Psychol Rep 43*, 1211–1217.
Hashimoto, K. (2009). Emerging role of glutamate in the pathophysiology of major depressive disorder. *Brain Res Rev 61*, 105–123.
Heider, F. (1958). *The Psychology of Interpersonal Relations*. New York: Wiley.
Hemingway, R., & Reigle, T. (1987). The involvement of endogenous opiate systems in learned helplessness and stress-induced analgesia. *Psychopharmacology 93*, 353–357.
Hiroto, D. S. (1974). Locus of control and learned helplessness. *J Exp Psychol*, 187–193.
Hunziker, M. H. L. (1992). Opioid nature of learned helplessness and stress-induced analgesia observed without re-exposure to shock. *Behav Pharmacol 3*, 117–121.
Hyman, S. E. (2007). Can neuroscience be integrated into the DSM-V? *Nature Rev Neurosci 8*, 725–732.
ICD-10 (1994). *International Statistical Classification of Diseases and Related Health Problems*. 10th Revision..
Ilin, Y., & Richter-Levin, G. (2009). ERK and CREB activation in the amygdala when an event is remembered as "fearful" and not when it is remembered as "instructive". *J Neurosci Res 87*, 1823–1831.
Ioannidis, J. P., Patsopoulos, N. A., & Rothstein, H. R. (2008). Reasons or excuses for avoiding meta-analysis in forest plots. *BMJ 336*, 1413–1415.
Jackson, R. L., Maier, S. F., & Rapaport, P. M. (1978). Exposure to inescapable shock produces both activity and associative deficits in the rat. *Learn Motiv 9*, 69–98.
Jacobs, N., Kenis, G., Peeters, F., Derom, C., Viletnick, R., & Van Os, J. (2006). Stress-related negative affectivity and genetically altered serotonin transporter function: evidence of synergism in shaping risk of depression. *Arch Gen Psychiatry 63*, 989–996.
Jankowski, M. P., & Sesack, S. R. (2004). Prefrontal cortical projections to the rat dorsal raphe nucleus: ultrastructural features and associations with serotonin and gamma-aminobutyric acid neurons. *J Comp Neurol 468*, 518–529.
Joca, S. R. L., Zanelati, T., & Guimaraes, F. S. (2006). Post-stress facilitation of serotonergic, but not noradrenergic, neurotransmission in the dorsal hippocampus prevents learned helplessness development in rats. *Brain Res 1087*, 67–74.
Joiner, T. E., Jr., Steer, R. A., Abramson, L. Y., Alloy, L. B., Metalsky, G. I., & Schmidt, N. B. (2001). Hopelessness depression as a distinct dimension of depressive symptoms among clinical and non-clinical samples. *Behav Res Ther 39*, 523–536.
Keller, M. C., Neale, M. C., & Kendler, K. S. (2007). Association of different adverse life events with distinct patterns of depressive symptoms. *Am J Psychiatry 164*, 1521–1529.
Kendler, K. S., & Gardner, C. O. (2010). Dependent stressful life events and prior depressive episodes in the prediction of major depression: the problem of causal inference in psychiatric epidemiology. *Arch Gen Psychiatry 67*, 1120–1127.
Kendler, K. S., Gardner, C. O., & Prescott, C. A. (2002). Toward a comprehensive development model for major depression in women. *Am J Psychiatry 159*, 1133–1145.
Kendler, K. S., Hettema, J. M., Butera, F., Gardner, C. O., & Prescott, C. A. (2003). Life event dimensions of loss, humiliation, entrapment, and danger in the prediction of onsets of major depression and generalized anxiety. *Arch Gen Psychiatry 60*, 789–796.
Kessler, R. C., Berglund, P., Demler, O., Jin, R., Merikangas, K. R., & Walters, E. E. (2005). Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. *Arch Gen Psychiatry 62*, 593–602.
Kessler, R. C., Birnbaum, H. G., Shahly, V., Bromet, E., Hwang, I., McLaughlin, K. A., et al. (2010). Age differences in the prevalence and co-morbidity of DSM-IV major depressive episodes: results from the WHO World Mental Health Survey Initiative. *Depress Anxiety 27*, 351–364.
Kessler, R. C., Chiu, W. T., Demler, O., Merikangas, K. R., & Walters, E. E. (2005). Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. *Arch Gen Psychiatry 62*, 617–627.
Kessler, R. C., Demler, O., Frank, R. G., Olfson, M., Pincus, H. A., Walters, E. E., et al. (2005). Prevalence and treatment of mental disorders, 1990 to 2003. *N Engl J Med 352*, 2515–2523.
Kessler, R. C., McLaughlin, K. A., Green, J. G., Gruber, M. J., Sampson, N. A., Zaslavsky, A. M., et al. (2010). Childhood adversities and adult psychopathology in the WHO World Mental Health Surveys. *Br J Psychiatry 197*, 378–385.
Koob, G. F. (1999). Corticotropin-releasing factor, norepinephrine, and stress. *Biol Psychiatry 46*, 1167–1180.
Korte, S. M., De Boer, S. F., & Bohus, B. (1999). Fear-potentiation in the elevated plus-maze test depends on stressor controllability and fear conditioning. *Stress 3*, 27–40.
Krishnan, V., & Nestler, E. J. (2008). The neurobiology of depression. *Nature 455*, 894–902.
Laidlaw, K., Davidson, K., Toner, H., Jackson, G., Clark, S., Law, J., et al. (2008). A randomised controlled trial of cognitive behaviour therapy vs treatment as usual in the treatment of mild to moderate late life depression. *Int J Geriatr Psychiatry 23*, 843–850.
Lefcourt, H. M. (1991). Locus of control. In J. P. Robinson, P. R. Shaver, & L. S. Wrightsman (Eds.), *Measures of Personality and Social Psychological Attitudes* (pp. 413–500). San Diego: Academic Press.
Liu, E. T. H., Chen, W. L., Li, Y. H., Wang, C. H., Mok, T. J., & Huang, H. S. (2009). Exploring the efficacy of cognitive bibliotherapy and a potential mechanism of change in the treatment of depressive symptoms among the Chinese: a randomized controlled trial. *Cogn Ther Res 33*, 449–461.
Liu, X., Tang, X., & Sanford, L. D. (2009). Stressor controllability and Fos expression in stress regulatory regions in mice. *Physiol Behav 97*, 321–326.
Lopez, A. D., Mathers, C. D., Ezzati, M., Jamison, D. T., & Murray, C. J. (2006). Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *Lancet 367*, 1747–1757.
Lynch, T. R., Morse, J. Q., Mendelson, T., & Robins, C. J. (2003). Dialectical behavior therapy for depressed older adults: a randomized pilot study. *Am J Geriatr Psychiatry 11*, 33–45.
Magalhaes, A. C., Holmes, K. D., Dale, L. B., Comps-Agrar, L., Lee, D., Yadav, P. N., et al. (2010). CRF receptor 1 regulates anxiety behavior via sensitization of 5-HT2 receptor signaling. *Nat Neurosci 13*, 622–629.
Maier, S. F., Grahn, R. E., Kalman, B. A., Sutton, L. C., Wiertelak, E. P., & Watkins, L. R. (1993). The role of the amygdala and dorsal raphe nucleus in mediating the behavioral consequences of inescapable shock. *Behav Neurosci 107*, 377–388.
Maier, S. F., & Seligman, M. E. P. (1976). Learned helplessness: theory and evidence. *J Exp Psychol*, 3–46.
Maier, S. F., & Watkins, L. R. (2005). Stressor controllability and learned helplessness: the roles of the dorsal raphe nucleus, serotonin, and corticotropin-releasing factor. *Neurosci Biobehav Rev 29*, 829–841.
Markou, A., Chiamulera, C., Geyer, M. A., Tricklebank, M., & Steckler, T. (2009). Removing obstacles in neuroscience drug discovery: the future path for animal models. *Neuropsychopharmacol 34*, 74–89.

Mathers, C. D., & Loncar, D. (2006). Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med 3*, e442.
McLaughlin, K. A., Green, J. G., Gruber, M. J., Sampson, N. A., Zaslavsky, A. M., & Kessler, R. C. (2010). Childhood adversities and adult psychiatric disorders in the national comorbidity survey replication II: associations with persistence of DSM-IV disorders. *Arch Gen Psychiatry 67*, 124–132.
Merikangas, K. R., He, J. P., Burstein, M., Swanson, S. A., Avenevoli, S., Cui, L., et al. (2010). Lifetime prevalence of mental disorders in U.S. adolescents: results from the National Comorbidity Survey Replication — Adolescent Supplement (NCS-A). *J Am Acad Child Adolesc Psychiatry 49*, 980–989.
Meyer, J. H., Houle, S., Sagrati, S., Carella, A., Hussey, D. F., Ginovart, N., et al. (2004). Brain serotonin transporter binding potential measured with carbon 11-labeled DASB positron emission tomography: effects of major depressive episodes and severity of dysfunctional attitudes. *Arch Gen Psychiatry 61*, 1271–1279.
Meyer, J. H., McMain, S., Kennedy, S., Korman, L., Brown, G., DaSilva, J., et al. (2003). Dysfunctional attitudes and serotonin2 receptors during depression and self harm. *Am J Psychiatry 160*, 90–99.
Millan, M. J. (2003). The neurobiology and control of anxious states. *Prog Neurobiol 70*, 83–244.
Miller, I. W., III, & Norman, W. (1979). Learned helplessness in humans: a review and attribution-theory model. *Psychol Bull 86*, 93–118.
Mo, B., Feng, N., Renner, K., & Foster, G. (2008). Restraint stress increases serotonin release in the central nucleus of the amygdala via activation of corticotropin-releasing factor receptors. *Brain Res Bull 76*, 493–498.
Moghaddam, B. (2002). Stress activation of glutamate neurotransmission in the prefrontal cortex: implications for dopamine-associated psychiatric disorders. *Biol Psychiatry 51*, 775–787.
Monroe, S. M., & Reid, M. W. (2008). Gene-environment interactions in depression research: genetic polymorphisms and life-stress polyprocedures. *Psychol Sci 19*, 947–956.
Nock, M. K., Borges, G., Bromet, E. J., Cha, C. B., Kessler, R. C., & Lee, S. (2008). Suicide and suicidal behavior. *Epidemiol Rev 30*, 133–154.
Nock, M. K., Hwang, I., Sampson, N., Kessler, R. C., Angermeyer, M., Beautrais, A., et al. (2009). Cross-national analysis of the associations among mental disorders and suicidal behavior: findings from the WHO World Mental Health Surveys. *PLoS Med 6*, e1000123.
Nolen-Hoeksema, S., Girgus, J. S., & Seligman, M. E. (1992). Predictors and consequences of childhood depressive symptoms: a 5-year longitudinal study. *J Abnorm Psychol 101*, 405–422.
Ongur, D., & Price, J. L. (2000). The organization of networks within the orbital and medial prefrontal cortex of rats, monkeys and humans. *Cereb Cortex 20*, 206–219.
Overmier, J. B., & Seligman, M. E. (1967). Effects of inescapable shock upon subsequent escape and avoidance responding. *J Comp Physiol Psychol 63*, 28–33.
Palermo-Neto, J., Massoco, C. D. O., & de Souza, W. R. (2003). Effects of physical and psychological stressors on behavior, macrophage activity, and Ehrlich tumor growth. *Brain Behav Immun 17*, 43–54.
Petersen, T. M., Pava, J. A., Buchin, J., Matthews, J. D., Papakostas, G. I., Nierenberg, A. A., et al. (2010). The role of cognitive–behavioral therapy and fluoxetine in prevention of recurrence of major depressive disorder. *Cognitive Therapy and Research 34*, 13–23.
Pezawas, L., Meyer-Lindenberg, A., Drabant, E. M., Verchinski, B. A., Munoz, K. E., Kolachana, B. S., et al. (2005). 5-HTTLPR polymorphism impacts human cingulate–amygdala interactions: a genetic susceptibility mechanism for depression. *Nature Neurosci 8*, 828–834.
Porsolt, R. D., Martin, P., Lenegre, A., Fromage, S., & Giurgea, C. E. (1989). Prevention of 'learned helplessness' in the rat by hydroxyzine. *Drug Dev Res 17*, 227–236.
Pryce, C. R., & Seifritz, E. (2011). A translational research framework for enhanced validity of mouse models of psychopathological states in depression. *Psychoneuroendocrinology 36*, 308–329.
Radloff, L. S. (1977). The CES-D Scale: a self-report depression scale for research in the general population. *Appl Psychol Meas 1*, 385–401.
Randich, A., & LoLordo, V. M. (1979). Associative and nonassociative theories of the UCS preexposure phenomenon: implications for Pavlovian conditioning. *Psychol Bull 86*, 523–548.
Rescorla, R. A., & Solomon, R. L. (1967). Two-process learning theory: relationships between Pavlovian conditioning and instrumental learning. *Psychol Rev 74*, 151–182.
Rolls, E. T. (2000). Précis of the brain and emotion. *Behav Brain Sci 23*, 177–234.
Rosellini, R. A., Warren, D. A., & DeCola, J. P. (1987). Predictability and controllability: differential effects upon contextual fear. *Learning and Motivation 18*, 392–420.
Rotter, J. B. (1966). Generalized expectancies for internal versus external control of reinforcement. *Psychol Monogr 80*, 1–28.
Salomons, T. V., Johnstone, T., Backonja, M. M., & Davidson, R. J. (2004). Perceived controllability modulates the neural response to pain. *J Neurosci 24*, 7199–7203.
Salomons, T. V., Johnstone, T., Backonja, M. M., Shackman, A. J., & Davidson, R. J. (2007). Individual differences in the effects of perceived controllability on pain perception: critical role of the prefrontal cortex. *J Cogn Neurosci 19*, 993–1003.
Sanacora, G., Zarate, C. A., Krystal, J. H., & Manji, H. K. (2008). Targeting the glutamatergic system to develop novel, improved therapeutics for mood disorders. *Nat Rev Drug Discov 7*, 426–437.
Schneider, F., Grodd, W., & Machulla, H. J. (1996). Examination of psychological functions by functional imaging with positron emission tomography and magnetic resonance imaging. *Nervenarzt 67*, 721–729.
Seedat, S., Scott, K. M., Angermeyer, M. C., Berglund, P., Bromet, E. J., Brugha, T. S., et al. (2009). Cross-national associations between gender and mental disorders in the World Health Organization World Mental Health Surveys. *Arch Gen Psychiatry 66*, 785–795.
Seligman, M. E. (1972). Learned helplessness. *Annu Rev Med 23*, 407–412.
Seligman, M. E., Castellon, C., Cacciola, J., Schulman, P., Luborsky, L., Ollove, M., et al. (1988). Explanatory style change during cognitive therapy for unipolar depression. *J Abnorm Psychol 97*, 13–18.
Seligman, M. E., & Maier, S. F. (1967). Failure to escape traumatic shock. *J Exp Psychol 74*, 1–9.
Seligman, M. E., Maier, S. F., & Geer, J. H. (1968). Alleviation of learned helplessness in the dog. *J Abnorm Psychol 73*, 256–262.
Seligman, M. E. P., Maier, S. F., & Solomon, R. L. (1971). Unpredictable and uncontrollable aversive events. In F. R. Black (Ed.), *Aversive Conditioning and Learning* (pp. 347–400). New York: Academic Press.
Sherman, A., & Petty, F. (1980). Neurochemical basis of the action of antidepressants on learned helplessness. *Behav Neural Biol 30*, 119–134.
Sims, J., Galea, M., Taylor, N., Dodd, K., Jespersen, S., Joubert, L., et al. (2009). Regenerate: assessing the feasibility of a strength-training program to enhance the physical and mental health of chronic post stroke patients with depression. *Int J Geriatr Psychiatry 24*, 76–83.
Singh, N. A., Stavrinos, T. M., Scarbek, Y., Galambos, G., Liber, C., & Fiatarone Singh, M. A. (2005). A randomized controlled trial of high versus low intensity weight training versus general practitioner care for clinical depression in older adults. *J Gerontol A Biol Sci Med Sci 60*, 768–776.
Smit, A., Tiemens, B. G., Ormel, J., Kluiter, H., Jenner, J. A., van der Meer, K., et al. (2005). Enhanced treatment for depression in primary care: first year results on compliance, self-efficacy, the use of antidepressants and contacts with the primary care physician. *Prim Care Community Psychiatry 10*, 39–49.
Spangler, D. L., Simons, A. D., Monroe, S. M., & Thase, M. E. (1993). Evaluating the hopelessness model of depression: diathesis-stress and symptom components. *J Abnorm Psychol 102*, 592–600.
Strigo, I. A., Simmons, A. N., Matthews, S. C., Craig, A. D., & Paulus, M. P. (2008). Association of major depressive disorder with altered functional brain response during anticipation and processing of heat pain. *Arch Gen Psychiatry 65*, 1275–1284.
Tan, H., Zhong, P., & Yan, Z. (2004). Corticotropin-releasing factor and acute stress prolongs serotonergic regulation of GABA transmission in prefrontal cortical pyramidal neurons. *J Neurosci 24*, 5000–5008.
Telner, J. I., & Singhal, R. L. (1981). Effects of nortriptyline treatment on learned helplessness in the rat. *Pharmacol Biochem Behav 14*, 823–826.
Toates, F. (1986). *Motivational Systems.* Cambridge: Cambridge University Press.
Tsang, H. W., Fung, K. M., Chan, A. S., Lee, G., & Chan, F. (2006). Effect of a qigong exercise programme on elderly with depression. *Int J Geriatr Psychiatry 21*, 890–897.
Vuorilehto, M. S., Melartin, T. K., & Isometsa, E. T. (2009). Course and outcome of depressive disorders in primary care: a prospective 18-month study. *Psychol Med 39*, 1697–1707.
Weiss, J. M., & Simson, P. G. (1986). Depression in an animal model: focus on the locus ceruleus. In C. F. Symposium (Ed.), *Antidepressants and Receptor Function Vol. 123*. (pp. 191–215)Chichester, U.K.: Wiley.
Weiss, J. M., et al. (1981). Behavioral depression produced by an uncontrollable stressor: relationship to norepinephrine, dopamine, and serotonin levels in various regions of rat brain. *Brain Res Rev 3*, 167–205.
Whitehouse, W. G., Blustein, J. E., Walker, J., Bersh, P. J., & Margules, D. L. (1985). Shock controllability and opioid substrates of escape performance and nociception: differential effects of peripherally and centrally acting naltrexone. *Behav Neurosci 99*, 717–733.
Willner, P. (1984). The validity of animal models of depression. *Psychopharmacology 83*, 1–16.

# Appendix 2

Neuropharmacology 62 (2012) 358–372

Contents lists available at SciVerse ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

# Establishing a learned-helplessness effect paradigm in C57BL/6 mice: Behavioural evidence for emotional, motivational and cognitive effects of aversive uncontrollability *per se*

Christopher R. Pryce [a,*], Damiano Azzinnari [a], Hannes Sigrist [a], Tilo Gschwind [a], Klaus-Peter Lesch [b], Erich Seifritz [a]

[a] *Preclinical Laboratory for Translational Research into Affective Disorders, Clinic for Affective Disorders & General Psychiatry, Psychiatric University Hospital Zürich, August Forel-Strasse 7, CH-8008 Zürich, Switzerland*
[b] *Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg, Würzburg, Germany*

ARTICLE INFO

*Article history:*
Received 22 April 2011
Received in revised form
4 August 2011
Accepted 6 August 2011

*Keywords:*
C57BL/6 mice
5-HTT
Two-way escape
Electro-shock US
CS–US conditioning
Learned aversive uncontrollability

ABSTRACT

Uncontrollability of major life events has been proposed to be central to depression onset and maintenance. The learned helplessness (LH) effect describes a deficit in terminating controllable aversive stimuli in individuals that experienced aversive stimuli as uncontrollable relative to individuals that experienced the same stimuli as controllable. The LH effect translates across species and therefore can provide an objective-valid readout in animal models of depression. Paradigms for a robust LH effect are established and currently applied in rat but there are few reports of prior and current study of the LH effect in mouse. This includes the C57BL/6 mouse, typically the strain of choice for application of molecular-genetic tools in pre-clinical depression research. The aims of this study were to develop a robust paradigm for the LH effect in BL/6 mice, provide evidence for underlying psychological processes, and study the effect of a depression-relevant genotype on the LH effect. The apparatus used for in/escapable electro-shock exposure and escape test was a two-way shuttle arena with continuous automated measurement of locomotion, compartment transfers, e-shock escapes, vertical activity and freezing. Brother-pairs of BL/6 mice were allocated to either escapable e-shocks (ES) or inescapable e-shocks (IS), with escape latencies of the ES brother used as e-shock durations for the IS brother. The standard two-way shuttle paradigm was modified: the central gate was replaced by a raised divider and e-shock escape required transfer to the distal part of the safe compartment. These refinements yielded reduced superstitious, pre-adaptive e-shock transfers in IS mice and thereby increased the LH effect. To obtain a robust LH effect in all brother pairs, pre-screening for minor between-brother ES differences was necessary and did not confound the LH effect. IS mice developed reduced motor responses to e-shock, consistent with a motivational deficit, and absence of a learning curve for escapes at escape test, consistent with a cognitive deficit. When a tone CS was used to predict e-shock, IS mice exhibited increased reactivity to the CS, consistent with hyper-emotionality. There was no ES–IS difference in pain sensitivity. Mice heterozygous knockout for the 5-HTT gene exhibited an increased LH effect relative to wildtype mice. This mouse model will allow for the detailed molecular study of the aetiology, psychology, neurobiology and neuropharmacology of uncontrollability of aversive stimuli, a potential major aetiological factor and state marker in depression.

This article is part of a Special Issue entitled 'Anxiety and Depression'.



## 1. Introduction

Helplessness, external locus of control and impaired coping ability, are inter-related and major concepts in depression, as predisposing, aetiological and pathological psychological processes (Abramson et al., 1989; Alloy et al., 1999; Harrow et al., 2009). These terms describe the individual's perceived lack of control over environmental events and, although they are not symptoms according to current diagnostic criteria, they are important state markers for therapy, both *per se* and as a potential route to ameliorate core and common depression symptoms such as depressed mood, fatigue, pessimistic views of the future and recurrent thoughts of suicide (DSM-IV, 1994; ICD-10, 1994).

* Corresponding author. Tel.: +41 44 634 8921; fax: +41 44 634 8829.
*E-mail addresses:* christopher.pryce@bli.uzh.ch (C.R. Pryce), damiano.azzinnari@bli.uzh.ch (D. Azzinnari), hannes.sigrist@bli.uzh.ch (H. Sigrist), tilo.gschwind@uzh.ch (T. Gschwind), kplesch@mail.uni-wuerzburg.de (K.-P. Lesch), erich.seifritz@bli.uzh.ch (E. Seifritz).

0028-3908/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuropharm.2011.08.012

Learned helplessness (LH) refers to the process in which the experiencing of major aversive life events that are uncontrollable (in terms of their outcome and termination) can lead to the individual responding to subsequent events as if they are also uncontrollable when in fact they are not (Abramson et al., 1978; Miller and Norman, 1979). Learned helplessness has been proposed to comprise the following three dimensions: emotional, in terms of increased arousal, anxiety and fear relative to stimuli/events; motivational, in terms of the reduced level of incentive to terminate the stimulus/event; cognitive, in terms of the reduced expectancy that the stimulus/event can be terminated by own behaviour (Maier and Seligman, 1976). In addition to embracing these three central dimensions of brain-behaviour function, LH is also distinguished by being the first example of a translational concept in psychiatric research. Thus, LH was first demonstrated in animal experimental psychology, including in dog and rat studies (Maier and Seligman, 1976), and the observations and interpretations made with these species were then applied to human behaviour and affective disorders thereof (Abramson et al., 1978; Miller and Norman, 1979; Seligman, 1972). Importantly, the LH effect demonstrated in animals is a deficit in responding to a specific stimulus/event (see below) and not a generalized helplessness, which is the form of helplessness regarded to be important in depression psychopathology (Abramson et al., 1978). Nonetheless, the LH effect model is important in terms of what it could reveal about the underlying neurobiology of specific and generalized helplessness, and therefore depression and its treatment (Amat et al., 2005; Robbins, 2005).

Most LH experiments have been conducted in the rat. Subjects are allocated at random either to exposure to controllable (escapable) or uncontrollable aversive stimuli and this is followed by a test in which all subjects are assessed in terms of their behaviour relative to controllable aversive stimuli. In the exposure phase, the operant latencies to terminate the escapable stimuli (ES) in the ES group are used as the durations of the inescapable stimuli (IS) in the IS group; as a result of this yoking principle the only difference between ES and IS is the controllability of the aversive stimuli experienced (Maier and Seligman, 1976). The current most commonly used rat LH model comprises exposure to tail electro-shock that can be terminated in the ES group by operant turning of a wheel, and testing in terms of escaping electro-shock by operant running in a two-way shuttle box: IS rats exhibit an escape deficit at test in terms of reduced number of escapes/increased latency to escape, where the escape response involves crossing from one side of the shuttle box to the other and back again (Amat et al., 2005). A distinguishing feature of the LH effect as a model in pre-clinical depression research is that the process underlying the behavioural change and being measured is clear: it is the prior experience of uncontrollability. This is not the case for other models: for example, the forced swim test is often claimed to measure "behavioural despair" (e.g., Castagné et al., 2011), "helplessness" (e.g. El Yacoubi et al., in press), "passive coping" (e.g. Keck et al., 2003) or even "active coping" (e.g. Lu et al., 2008) but, because there is no equivalent of the ES comparison group in the LH effect paradigm, it is difficult to interpret what underlies the reduction in swimming exhibited by rats and mice and, therefore, what the test actually measures.

Despite the high use and potential of mice in pre-clinical research into affective disorders, and despite the high face and construct validity of the LH effect paradigm, there have been relatively few published studies of a mouse LH effect. A recent literature search (Embase, Medline and Psycinfo, from 1806 to 2011) of rat and mouse LH effect studies that used the ES–IS paradigm yielded 38 rat studies and 6 mouse studies (Pryce et al., in press) and the most recent mouse study was published in 2003 (Palermo-Neto et al., 2003). There are a large number of mouse publications which include comparison of an IS group with a group that received no aversive stimuli prior to test, the so-called unconditioned-stimulus (US) pre-exposure effect paradigm (e.g. Chang et al., 2007; Maeng et al., 2008). These studies do not allow for assessment of the effect of aversive uncontrollability *per se* and thereby lack the very essence of the LH effect paradigm. One laboratory that has made a significant contribution to establishing and studying the LH effect in mice is that of Anisman, with the majority of studies conducted with Swiss-Webster mice and using electro-shocks for ES–IS pre-exposure and e-shock two-way escape behaviour as readout (e.g. Anisman et al., 1978, 1979, 1980; Anisman and Merali, 2001). As noted (Anisman and Merali, 2001), one possible reason for the relative scarcity of mouse LH effect studies is the challenge of designing an aversive manipulation such that those mice pre-exposed to the escapable form (ES) continue to exhibit high levels of escape behaviour up to and including the escape test, whilst those mice pre-exposed to the inescapable form of the manipulation (IS) – which is identical in intensity and duration to ES – develop a robust escape deficit that is expressed during the escape test.

Given its common application in molecular-genetic models in pre-clinical depression research, the first aim of the current study was to establish an LH effect paradigm in the C57BL/6 mouse strain. Both ES–IS pre-exposure and escape test were carried out using a two-way e-shock escape apparatus that allowed for concurrent data collection on several relevant behavioural measures. The apparatus was modified across experiments to increase the robustness of the LH effect. The experimental design was based on brother-pairs, such that each ES–IS dyad was also a brother-pair. This was commensurate with the yoked design of the ES–IS paradigm and, given that inter-individual differences are pronounced even within inbred mouse strains such as C57BL/6 (e.g. Siegmund et al., 2009), using brother pairs could also reduce existing individual (trait) differences between mice allocated to the same ES–IS dyad and thereby further increase the robustness of the LH effect obtained. The second aim of the study was to use features and behavioural measures provided by the apparatus to identify the extent to which emotional, motivational and cognitive processes contributed to the LH effect. The third aim was to study the effects of heterozygote knockout (HET) of the serotonin transporter gene (Slc6a4, commonly abbreviated to 5-HTT) on the LH effect. Reduced 5-HTT activity in human due to the *short* variant of the 5-HTT gene-linked polymorphic region is associated with a relatively high risk of depression in individuals exposed to several aversive life events (Caspi et al., 2003; Karg et al., 2011). In mouse, 5-HTT HET mice exhibit reduced two-way escape responding following IS relative to wildtype in the US pre-exposure effect paradigm (Muller et al., 2011). An increased LH effect in 5-HTT HET mice would demonstrate that this phenotype is due to increased uncontrollability and demonstrate the translational validity of the mouse LH effect for neuro-biological and –pharmacological study of depression and its treatment.

## 2. Materials and methods

### 2.1. Animals and maintenance

Male C57BL/6J mice (RCC, Füllinsdorf, Basel, Switzerland) were delivered to the laboratory at age 8 weeks and allowed 2 weeks to adjust to a reversed light–dark cycle (lights off at 07:00–19:00 h). Male and female 5-HTT knockout mice on a C57BL/6J background (>20 backcross generations) were transferred from the University of Würzburg (Bengel et al., 1998) and breeding was established in-house with wildtype (WT) dams and 5-HTT heterozygote knockout (HET) sires. Study mice were weaned at 4 weeks and genotyped.

Mice were held in individually-ventilated cages (type 2 long) containing sawdust, a sleeping house and bedding, with continuous access to food and water.

Mice were housed as brother-pairs, each pair from a different litter and of the same genotype in the case of the 5-HTT strain, and were maintained in these pairs for 10 days (C57BL/6J) or 6 weeks (5-HTT). Four days before the onset of the experiments, mice were singly housed in new cages of the same type and under the same conditions; they remained singly housed throughout the experiment.

All behavioural testing was conducted in a dimly lit room adjacent to the mouse holding room. Each separate experiment was conducted with naive mice.

All procedures were conducted under a permit for animal experimentation issued by the Veterinary Office, Zurich, Switzerland, in accordance with the Animal Protection Act (1978) Switzerland. All efforts were made to minimise the number of mice used and any suffering of those mice that were used (see also Discussion for relevance of paradigm developed to the 3R's).

### 2.2. Hot plate test

Mice were given two hot plate tests to assess pain sensitivity, one at 3 days prior to the onset of the LH-effect experiment and one at 1 day after its completion. The hot plate test was conducted using a programmable thermoelectric heating plate (Teca, Chicago IL, USA) set at 50 °C, with a transparent Plexiglas chamber plus removable lid fixed onto the plate. All hot plate tests were conducted between 15:00–17:00 h. The mouse was removed from the home cage, weighed, placed inside the chamber and the lid closed. The latency (seconds) from the onset of the test until the first occurrence of one of the following behaviours was scored: licking a forepaw, licking a hind paw, lifting a hind paw, jumping. The mouse was then immediately removed from the hot plate. The maximum test duration was 60 s and 60 s was the latency score given to mice that did not exhibit one of the target behaviours.

### 2.3. Learned helplessness effect experiments

Electro-shock (e-shock) stimulation and behavioural assessment were conducted using a fully-automated apparatus, specific hardware and software features of which were purpose-built/-written for this study (Multi Conditioning System, TSE Systems GmbH, Bad-Homburg, Germany) (Fig. 1). An e-shock grid floor measuring 30 × 30 cm comprising 29 stainless steel rods (Ø = 4 mm, inter-rod centre-to-centre distance = 10 mm) was fixed in a frame. A quadratic arena made out of dark Plexiglas on three sides and transparent Plexiglas at the front, measuring 30 × 30 × 24 (H) cm, was positioned on top of the grid. Running from front to back of the arena and situated at its midline, was either: (i) a dark Plexiglas wall containing an opening ("gate") at its midline with dimensions 3.5 (W) × 10.0 (H) cm, allowing the mouse to transfer from one compartment of the arena/grid to the other through the gate; (ii) a transparent Plexiglas divider: except for two 1.5 cm-wide feet extending down to just above the grid at both ends, the bottom edge of the divider was 2.5 cm above the grid, allowing for transfer of the mouse from one side of the arena/grid to the other across nearly the entire depth of the arena/grid. The frame surrounding the arena/grid included three infrared light-beam sensor systems with sensors spaced 14 mm apart. Two of these systems were for movement detection in the X–Y horizontal plane, and allowed measurement of quadrupedal locomotion distance, number of transfers from one arena side to the other, and number and duration of freezing episodes, defined as no detection of any movement for a minimum of 2 s. The third sensor system, placed on top of the Y system, was for movement detection in the Z vertical plane, and number of rears (XYZ bipedal standing), climbs (XYZ bipedal leaning and upward movements against an arena surface) and jumps (Z detection only) were scored with this system. Whilst full jumps could be measured unambiguously, some responses scored as rears and climbs were actually low jumps and therefore a composite score of rears/climbs/jumps was used. A transparent Plexiglas lid was placed on top of the arena, and a metal waste tray was positioned underneath the grid floor. This apparatus was contained in an attenuating chamber, equipped with a ventilation fan, house lights (set to provide 8 lux), a loud speaker emitting low-level white noise, and an observation window. Four such units were used, interfaced with a dedicated control unit, which in turn was connected to a PC running control and data collection software. Although all data collection was automated, all sessions were also observed directly by the experimenters.

Four experiments were conducted. In experiment I (Standard conditions), the gate was used, transfer through the gate terminated the e-shock immediately, and mice in brother-pairs ($N = 12$) were allocated to groups at random, one to the escapable e-shock (ES) group and one to the inescapable e-shock (IS) group. In experiment II (Refined conditions, Random allocation), the divider was used, termination of the e-shock was not physically or temporally coincident with transfer under the divider (see below), and mice in brother-pairs ($N = 12$) were allocated to the ES and IS groups at random. In experiment III (Refined conditions, Screening allocation), the divider was used, termination of the e-shock was not physically–temporally coincident with transfer under the divider, and mice in brother-pairs ($N = 17$) were allocated to the ES and IS groups based on their relative escape performance during an initial ES session (screening). An additional experiment was conducted to attempt to validate the screening procedure. In experiment IV (5-HTT HET versus WT, Refined conditions, Screening allocation), the conditions were the same as in experiment III and mice were in brother-pairs of WT–WT ($N = 8$) or HET–HET ($N = 8$). The specific protocols for the four experiments are detailed below. General conditions were: All sessions were conducted between 08:30–12:00 h. After each session on each day, the arenas were removed and wiped with 70% ethanol. The grids were wiped dry of any urine with dry absorbent paper and then wiped with 70% ethanol. The waste tray was removed, rinsed with hot water and wiped dry with absorbent paper. The correct functioning of the grids and the movement detection system was controlled prior to running each group of four mice.

#### 2.3.1. Experiment I: Standard conditions

Day 1, *Habituation*: The mouse was removed from its home cage, weighed, and placed on the grid in the arena. The gate was used as the central divider. A 15-min session was started during which the mouse could locomote in and explore the arena. No e-shocks were administered.

Day 2, *Paired ES–IS pre-exposure 1*: The mouse allocated at random to the ES group was placed in the arena for 2-min habituation. ES mice were exposed to 30 e-shocks each of 0.15 mA amplitude and 5-s maximum duration on a 50-s constant inter-trial interval (ITI). The gate was used as the central divider. Parameters were set so that 100% of the grid-side on which the mouse was standing emitted e-shock and 100% of the opposite grid-side was "safe for escape", such that as soon as the mouse transferred once through the gate the e-shock was terminated and the next ITI was initiated. Mice could transfer through the gate at any time. For each trial, the grid-side on which the mouse was positioned at the onset of e-shock was the aversive side and the opposite side the escape side. Therefore, the aversive side and escape side on each trial were

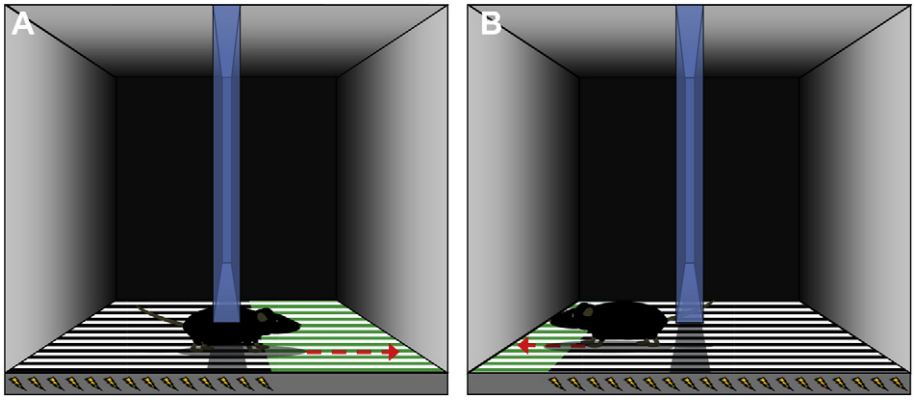



**Fig. 1.** A schematic of the experimental set-up. Mice were placed on a floor of electrifiable stainless steel rods in an arena with three solid walls and a transparent front wall, and with a central divider running from back to front and raised off the ground. The mouse could pass under the central divider. (A) The sketch depicts an ES mouse in Pre-exposure 1 in Experiments II–IV. The mouse is in compartment Left and the e-shock is presented Left and Right. Transfer of the mouse under the central divider and crossing with the head beyond the first 25% of the Right compartment results in termination of the e-shock. (B) The sketch depicts an ES mouse in Pre-exposure 2 or 3 or an ES or IS mouse in Escape test, in Experiments II–IV. The mouse is in compartment Right and the e-shock is presented Right and Left. Transfer of the mouse under the central divider and crossing with the head beyond the first 70% of the Left compartment results in termination of the e-shock.

determined by the mouse. ES mice received 30 escapable e-shocks in total and the maximum possible e-shock exposure was 150 s. The total duration of the session was 22 min. The IS session, with the brother of the ES mouse, commenced after the ES session was completed: the e-shock escape latencies of the ES brother were used one-to-one as the durations for inescapable e-shock exposures for the IS brother. That is, the yoked design used traditionally in ES–IS studies (Maier and Seligman, 1976) was changed to a successive paired design. ES and IS brothers were run in the same apparatus unit.

Day 3, *Paired ES–IS pre-exposure 2*: The conditions were the same as for ES–IS pre-exposure 1.

Day 4, *Paired ES–IS pre-exposure 3*: The conditions were the same as for ES–IS pre-exposures 1–2 except the total number of trials was 24, the maximum duration per shock was 4 s, and the total duration of the session was 18 min.

Day 5: No session

Day 6, *Escape test*: Following a 48-h period during which ES and IS mice remained undisturbed in their home cages, the mice were placed in the arena for a session identical to ES pre-exposure 3, except that the e-shock was now escapable for ES and IS mice.

#### 2.3.2. Experiment II: Refined conditions, Random allocation

The experimental parameters were the same as for Experiment I, with the exception of the following changes.

Day 1, *Habituation*: The divider was used.

Day 2, *Paired ES–IS pre-exposure 1*: The mouse allocated to the ES group was placed in the arena for 2-min habituation. The divider was used. ES mice were exposed to 30 e-shocks each of 0.15 mA amplitude and 5-s maximum duration on a 50-s constant inter-trial interval (ITI). For each trial, the grid-side on which the mouse was positioned at the onset of e-shock was the aversive side and the opposite side the escape side. For e-shocks 1–10, 100% of the grid-side on which the mouse was standing emitted shock and the most distant 75% of the opposite grid-side was "safe", such that if the mouse passed under the divider and entered this 75% safe area the e-shock was terminated and the next ITI initiated (Fig. 1A). For e-shocks 11–20, 100% of the grid-side on which the mouse was standing emitted e-shock and the most distant 50% of the opposite grid-side was "safe" such that if the mouse passed under the divider and entered this 50% safe area the e-shock was terminated and the next ITI initiated. For e-shocks 21–30, 100% of the grid-side on which the mouse was standing emitted e-shock and the most distant 30% of the opposite grid-side was "safe" such that if the mouse passed under the divider and entered this 30% safe area the e-shock was terminated and the next ITI initiated (Fig. 1B). The IS session, with the brother of the ES mouse, commenced after the ES session was completed, so that the e-shock escape latencies of the ES brother were used one-to-one as the durations for inescapable e-shock exposures for the IS brother.

Day 3, *Paired ES–IS pre-exposure 2*: The conditions were the same as for ES–IS pre-exposure 1 except that the safe area of the opposite grid was 50% for e-shocks 1–10 and 30% for e-shocks 11–30.

Day 4, *Paired ES–IS pre-exposure 3*: The conditions were the same as for ES–IS pre-exposure 2 except for the following: the total number of trials was 24, the maximum duration per e-shock was 4 s, and the safe area of the opposite grid was 30% for all e-shocks.

Day 5: No session.

Day 6, *Escape test*: Following a 48-h period during which ES and IS mice remained undisturbed in their home cages, the mice were placed in the arena for a session identical to ES pre-exposure 3, except that the e-shock was now escapable for ES and IS mice.

#### 2.3.3. Experiment III: Refined conditions, Screening allocation

The experimental parameters were the same as for Experiment II, with the exception of the following changes.

Day 2, *ES Screening*: Both mice of each brother-pair were given ES pre-exposure 1 as described above for the ES group in Experiment II i.e. there was no IS condition. The brother with the lower mean escape latency to e-shocks 21–30 (and therefore lower total duration of e-shock exposure on trials 21–30) was allocated to the ES group and the other brother was allocated to the IS group.

Day 3, *Paired ES–IS pre-exposure 1* (not 2, otherwise identical to Day 3 in Experiment II).

Day 4, *Paired ES–IS pre-exposure 2* (not 3, otherwise identical to Day 4 in Experiment II).

Day 9, *CS-uncontrollable e-shock conditioning*: Following the LH procedure on days 1–6, this test was carried out in 11 of the 17 brother-pairs. In addition, 8 mice that had experienced the Habituation session only (i.e. naive to e-shock, NS) were also tested. Following a 72-h period during which ES, IS and NS mice remained undisturbed in their home cages, they were placed in the arena and after 2-min habituation were exposed to pairings of a tone (5 kHz, 85 dB, emitted from a loudspeaker situated above the centre of the arena) and unavoidable/inescapable e-shock. Each tone was of 14-s duration and the final 4 s were paired with a 0.15 mA × 4-s e-shock. Sixty such pairings were given with a constant ITI of 50 s. The total duration of the session was 65 min. The e-shock was uncontrollable to ensure that all mice received equal exposure to CSs, e-shocks, and CS − e-shock pairings. It was hypothesized that if the prior chronic experience of the (un)controllability of e-shock is of emotional valence to mice, then neutral stimuli that predict e-shock will, at least initially, elicit different behavioural responses from ES and IS mice. The NS group was necessary *a priori* to facilitate interpretation of any ES–IS differences. Locomotor distance, number of rears/climbs/jumps and time spent freezing, during ITI's and CS's, were of particular interest.

In a validation experiment for the screening procedure, both brothers ($N = 10$ pairs) went through ES screening, ES Pre-exposures 1 and 2, and Escape test i.e. there was no IS group. The aim was to investigate whether the mice with higher total duration of e-shock exposure on trials 21–30 (i.e. the criterion for allocation to IS) exhibited reduced escape behaviour at escape test even in the absence of IS pre-exposure. If this was the case then clearly the LH effect could not be inferred as a mediating mechanism when the ES Screening method was used.

#### 2.3.4. Experiment IV: Refined conditions, Screening allocation in 5-HTT knockout mice

Brother-pairs had the same genotype i.e. WT–WT, HET–HET; using mixed-genotype brother-pairs would have been problematic if one genotype consistently exhibited a lower mean escape latency at ES screening, because the distribution of genotypes across ES and IS would then not have been counter-balanced. The experimental parameters were the same as for Experiment III on Day 1–6, with the exception of the following changes.

Day 2, *ES Screening*: As in Experiment III, both mice of each brother-pair were given ES pre-exposure and there was no IS condition. Because pilot studies demonstrated that the 5-HTT ko strain developed more escape failure across daily sessions of 30 trials of escapable e-shock (ES) in comparison with the C57BL/6J strain, less trials were used. Thus, mice were exposed to 24 e-shock trials rather than 30. For e-shocks 1–8, 100% of the grid-side on which the mouse was standing emitted shock and the most distant 75% of the opposite grid-side was "safe"; for e-shocks 9–16, 100% of the grid-side on which the mouse was standing emitted e-shock and the most distant 50% of the opposite grid-side was "safe"; for e-shocks 17–24, 100% of the grid-side on which the mouse was standing emitted e-shock and the most distant 30% of the opposite grid-side was "safe" (Fig. 1B).

Day 3, *Paired ES–IS pre-exposure 1*: Again 24 rather than 30 e-shock trials were used. The safe area of the opposite grid was 50% for e-shocks 1–8 and 30% for e-shocks 9–24.

### 2.4. Statistical analysis

All statistical analyses were conducted using SPSS (version 17, SPSS Inc., Chicago IL, USA). For body weight and hot plate test, conducted both pre- and post-e-shock, Group (ES, IS) and Time (pre-, post-) were included in ANOVA as between- and within-subject factors, respectively. For LH effect experiments I–III, data for each behavioural measure per session were analysed for ES–IS brother-pairs using the paired *t*-test. In Experiment III, validation statistics were conducted to investigate whether there were behavioural differences between ES and IS mice at ES Screening on Day 2 that predicted escape responses at Escape test on Day 6. The most relevant ES Screening measures were the total duration of e-shock exposure (directly proportional to mean escape latency) on trials 21–30 i.e. the measure used to allocate each brother per pair to ES or IS, and total duration of e-shock exposure on trials 1–30. Any predictive associations would confound the interpretation that differences in ES and IS mice at Escape test were attributable primarily to their differential treatment at Pre-exposures 2 and 3. Firstly, regression analysis was conducted to investigate whether, within either the ES or IS group, inter-individual differences in Escape-test escape responses correlated significantly with either of the ES-Screening measures. Second, each of the ES Screening measures was included as the covariate in ANCOVA with the between-subject factor of Group (ES, IS) and the dependent variable of Escape test escape responses. To ensure that these validation statistics were sufficiently powered, a relatively large sample size of 17 brother-pairs was included in Experiment III. The significance of the regression was based on the *F*-distribution and correlations were calculated using Pearson's *r*. To analyse the effect of Group (ES, IS and NS) on behavioural scores in the CS-uncontrollable e-shock test, analysis of variance (ANOVA) was conducted. To analyse the effect of Genotype (WT, HET) and Group (ES, IS) on behavioural scores per session in Experiment IV, factorial ANOVAs were conducted. All *post hoc* testing was conducted using the Bonferroni correction for multiple comparisons. Significance was set at $p < 0.05$. All data are presented as mean ± standard deviation (SD).

## 3. Results

### 3.1. Experiment I: Standard conditions

In brother pairs allocated randomly to the groups ES and IS, there were no behavioural differences during Habituation or Pre-exposure 1. During Pre-exposures 2 and 3, there were consistent ES–IS differences in e-shock escapes/transfers: at Pre-exposure 2, ES mice exhibited more escapes than IS mice exhibited e-shock transfers ($t = 3.09$, $df = 11$, $p < 0.02$). This effect was increased at Pre-exposure 3 ($t = 3.46$, $df = 11$, $p < 0.006$; Fig. 2A). During the Escape test, there was a borderline non-significant difference in the number of escape responses of ES and IS mice ($t = 2.18$, $p < 0.06$; Fig. 2B) and no significant difference in the mean escape latency (ES: 2.5 ± 0.9 s, IS: 3.2 ± 0.8 s; $t = -1.58$, $p < 0.11$; Fig. 2B). ES mice made a similar number of escape responses in Pre-exposure 3 and Escape test ($t = -0.22$, $p < 0.81$) and within ES mice number of escape responses at Pre-exposure 3 predicted number of escape responses at Escape test ($r = 0.98$, $N = 12$, $p < 0.0005$). IS mice made significantly more escape responses at Escape test than transfers at Pre-exposure 3 ($t = -3.25$, $p < 0.009$), and within IS mice number of transfers at Pre-exposure 3 predicted number of escape responses at test ($r = 0.97$, $N = 12$, $p < 0.0005$). As evident in Fig. 2, there was marked intra-group variability in both ES and IS mice during both Pre-exposure 3 and Escape test: in the two ES mice that made the fewest escape responses, their IS brothers made more transfers/escape responses, and despite long inescapable e-shock pre-exposures.

Data for pain response latency in the hot plate test and body weight are given in Table 1. There was no effect of Group or Time on pain response latency or body weight.

Given that (i) the LH effect observed was borderline and, in IS mice, (ii) there were more e-shock escapes at test than e-shock transfers at Pre-exposure 3 and (iii) transfers at Pre-exposure 3 predicted e-stimulus escapes at Escape test, the apparatus was modified in an attempt to reduce e-shock transfers by IS mice during pre-exposure sessions. It was hypothesized that replacement of the gate with a divider would reduce the number of e-stimulus transfers and therefore subsequent escapes in the IS condition whilst not affecting escapes in the ES group. This hypothesis was tested in Experiment II.

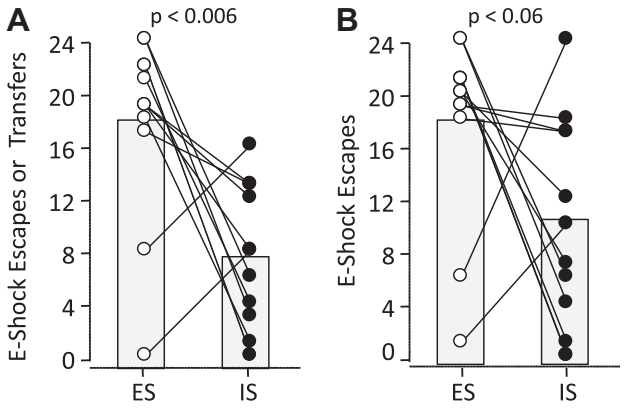



**Fig. 2.** E-shock escape and transfer data (individual values for 12 brother-pairs and group means) for Experiment I, Standard conditions. (A) Pre-exposure session 3: number of escapes by ES mice and transfers by IS mice in response to 24 × 4-s maximum × 0.15 mA e-shocks. (B) Escape test: number of escapes by ES mice and IS mice in response to 24 × 4-s maximum × 0.15 mA e-shocks. *P* values are for paired *t*-tests.

**Table 1**
Data summary for body weight and hot plate behaviour pre- and post-LH effect testing in Experiments I–IV.

| Parameter | | ES | | IS | |
|---|---|---|---|---|---|
| | | Pre-Test | Post-Test | Pre-Test | Post-Test |
| Experiment I, $N = 12$ pairs | | | | | |
| Body weight (g) | X | 24.7 | 25.3 | 25.3 | 24.8 |
| | SD | 1.4 | 1.0 | 0.7 | 1.2 |
| Pain response latency (sec) | X | 32.1 | 26.2 | 25.8 | 33.4 |
| | SD | 15.0 | 14.8 | 11.4 | 8.7 |
| Experiment II, $N = 12$ pairs | | | | | |
| Body weight (g) | X | 26.6 | 27.1 | 26.9 | 26.9 |
| | SD | 2.1 | 1.8 | 1.3 | 1.5 |
| | | Group × Time $p < 0.06$ | | | |
| Pain response latency (sec) | X | 30.3 | 39.7 | 31.8 | 36.9 |
| | SD | 10.3 | 10.3 | 6.7 | 17.7 |
| | | Time $p < 0.04$ | | | |
| Experiment III, $N = 17$ pairs | | | | | |
| Body weight (g) | X | 26.8 | 26.7 | 27.2 | 27.4 |
| | SD | 1.9 | 1.8 | 2.5 | 2.1 |
| Pain response latency (sec) | X | 37.4 | 30.5 | 33.0 | 35.6 |
| | SD | 11.8 | 11.1 | 11.6 | 10.0 |
| | | Group × Time $p < 0.04$ | | | |
| Experiment IV, $N = 8, 8$ pairs | | | | | |
| Body weight (g) | | | | | |
| WT | X | 27.2 | 25.6 | 26.0 | 26.2 |
| | SD | 3.7 | 1.2 | 1.5 | 1.3 |
| HET | X | 25.3 | 25.6 | 23.7 | 23.1 |
| | SD | 2.8 | 2.4 | 6.6 | 8.5 |
| Pain response latency (sec) | | | | | |
| WT | X | 32.4 | 30.6 | 35.0 | 25.8 |
| | SD | 13.1 | 12.2 | 6.3 | 9.8 |
| HET | X | 29.3 | 30.0 | 25.2 | 27.4 |
| | SD | 9.0 | 6.8 | 15.6 | 16.4 |

### 3.2. Experiment II: Refined conditions, Random allocation

In brother pairs allocated randomly to the groups ES and IS, there were no ES–IS behavioural differences during Habituation. During e-shock pre-exposure sessions, escapes/transfers was the single parameter that exhibited consistent ES–IS group differences. Thus, at Pre-exposure 1, ES mice exhibited more escapes than IS mice exhibited e-shock transfers ($t = 6.76$, $df = 11$, $p < 0.0005$; Fig. 3A), and this effect persisted during Pre-exposure 2 ($t = 4.05$, $p < 0.003$) and Pre-exposure 3 ($t = 2.55$, $p < 0.04$; Fig. 3B). During the Escape test, IS mice exhibited significantly fewer escapes than did ES mice ($t = -2.83$, $p < 0.03$; Fig. 3C), and had a significantly longer mean escape latency (ES: 2.6 ± 0.8 s, IS: 3.6 ± 0.4 s; $t = 2.69$, $p < 0.03$). The ES mice made a similar number of escape responses in Pre-exposure 3 and Escape test ($t = -1.97$, $p < 0.09$) and within ES mice number of escape responses at Pre-exposure 3 predicted number of escape responses at Escape test ($r = 0.96$, $N = 12$, $p < 0.0005$; Fig. 3D). IS mice made a similar number of escape responses at Escape test to transfers at Pre-exposure 3 ($t = -0.42$, $p = 0.68$), and within IS mice number of transfers at Pre-exposure 3 predicted number of escape responses at test ($r = 0.84$, $N = 12$, $p < 0.0005$; Fig. 3E). As evident in Fig. 3, there was marked intra-group variability in escape responding in both ES and IS. Furthermore, in the three brother-pairs where the ES mouse exhibited few escapes, the IS brother (i) exhibited more escapes, despite long inescapable e-shock pre-exposures, and (ii) exhibited the highest numbers of escape responses within the IS group.

Data for pain response latencies in the hot plate test and body weight are given in Table 1. There was a significant main effect of Time on pain response latency ($F(1, 22) = 5.32$, $p < 0.04$), with latencies being greater at Post-Escape test. *A posteriori* analysis of ES-Pre, ES-Post, IS-Pre and IS-Post did not yield a significant effect ($F(1, 22) = 2.89$, $p < 0.09$). For body weight, there was a borderline non-significant interaction effect of Group × Time ($F(1, 22) = 4.49$,

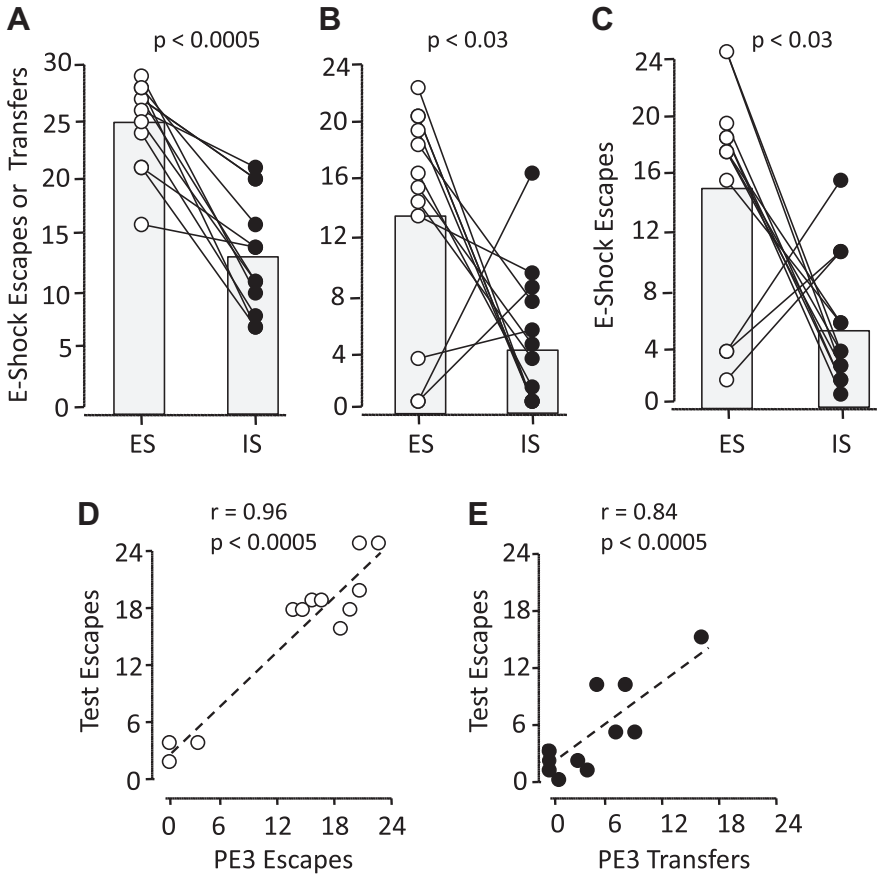



**Fig. 3.** E-shock escape and transfer data (individual values for 12 brother-pairs and group means) for Experiment II, Refined conditions, Random allocation. (A) Pre-exposure session 1: number of escapes by ES mice and transfers by IS mice in response to 30 × 5-s maximum × 0.15 mA e-shocks, with 10 trials each at 75%, 50% 30% safe area in the escape compartment. (B) Pre-exposure session 3: number of escapes by ES mice and transfers by IS mice in response to 24 × 4-s maximum × 0.15 mA e-shocks, with 30% safe area in the escape compartment. (C) Escape test: number of escapes by ES mice and IS mice in response to 24 × 4-s maximum × 0.15 mA e-shocks. A–C: *p* values are for paired *t*-tests. (D) Scattergram, regression line and associated probability, and Pearson's *r* for Pre-exposure 3 escapes versus Test escape responses in ES mice. (E) Scattergram, regression line and associated probability, and Pearson's *r* for Pre-exposure 3 escapes versus Test escape responses in IS mice.

$p < 0.06$). *A posteriori* analysis also yielded a significant effect ($F(2, 22) = 4.98$, $p < 0.03$), which was due to increased body weight in ES mice at Post- versus Pre-Escape test ($p < 0.04$).

In contrast to the gate, using the divider there was no increase in escapes at Escape test relative to transfers at Pre-exposure 3 in the IS group. In both Experiments I and II there was a minority of brother-pairs in which the mouse allocated at random to ES made few escapes relative to other ES mice and its IS brother, and the latter made a high number of escapes relative to other IS mice and its ES brother. This led to high intra-group variability in both ES and IS groups. It was hypothesized that exposing both brothers to a screening session in which they both received ES and then allocating the brother with the shorter mean escape latency (lower total e-shock exposure) in the final third of the screening session to the ES group, would reduce intra-group variability. This hypothesis was tested and the method validated in Experiment III.

### 3.3. Experiment III: Refined conditions, ES Screening allocation

Retrospective analysis demonstrated that there were no "ES"–"IS" behavioural differences during Habituation, including ITI distance moved (Table 2). At ES Screening, both brothers per pair were exposed to escapable e-shocks and the brother that exhibited the lower mean escape latency (and therefore lower total e-shock exposure) at trials 21–30 was allocated to the ES group and *vice versa*. Across trials 1–30, "ES" mice made significantly more e-stimulus escapes than "IS" mice ($t = 2.83$, $df = 16$, $p < 0.03$; Fig. 4A, Table 2) and mean escape latency was significantly shorter in "ES" mice than in "IS" mice ($t = -2.80$, $p = 0.02$; Table 2). Across trials 1–20, e-stimulus escapes were not significantly different in "ES" and "IS" mice ("ES" 17 ± 3, "IS" 16 ± 3, $p < 0.80$) and nor were mean escape latencies ("ES" 1.9 ± 0.7, "IS" 2.3 ± 0.8 s, $p < 0.12$). Across trials 21–30, "ES" mice made 9 ± 1 escape responses with a mean escape latency of 2.0 ± 0.8 s and "IS" mice made 7 ± 2 escape responses with a mean escape latency of 2.9 ± 0.8 s.

At Pre-exposure 2, ES mice exhibited significantly more e-shock escapes than IS mice exhibited e-shock transfers ($t = 11.25$, $p < 0.0005$; Fig. 4B). The IS mice exhibited significantly shorter e-shock distance moved ($t = 3.64$, $p < 0.003$), and significantly greater ITI distance moved ($t = -3.10$, $p < 0.008$) (Table 2). At Pre-exposure 3, ES mice exhibited significantly more e-shock escapes than IS mice exhibited e-shock transfers ($t = 11.82$, $p < 0.0005$; Fig. 4C). The IS mice exhibited significantly shorter e-shock distance moved ($t = -3.68$, $p < 0.003$), significantly greater ITI distance moved ($t = 4.62$, $p < 0.0005$), significantly more ITI transfers ($t = 4.91$, $p < 0.0005$), significantly more ITI rears/climbs/jumps ($t = 3.16$, $p < 0.007$) and significantly less ITI % time freezing ($t = -3.10$, $p < 0.008$) (Table 2). At Escape test, IS mice exhibited significantly fewer e-shock escapes than ES mice ($t = -11.86$, $p < 0.0005$; Fig. 4D) and a significantly longer mean e-shock escape latency ($t = 8.29$, $p < 0.0005$; Fig. 4D). The IS mice also exhibited a significantly shorter e-shock distance moved ($t = -4.73$,

**Table 2**
Summary table for experiment III, in which brother-pairs were allocated to ES and IS treatment groups following screening.

| Parameter | | Habituation (15′) | | Pre-exposure 1 (22.5′, 30 trials) | | Pre-exposure 2 (22.5′, 30 trials) | | Pre-exposure 3 (18′, 24 trials) | | Escape test (18′, 24 trials) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ES | IS | ES | IS | ES | IS | ES | IS | ES | IS |
| E-shock, ES Escapes/IS Transfers (Total) | X | | | 26 | 23* | 24 | 10**** | 20 | 4**** | 18 | 5**** |
| | SD | | | 3 | 4 | 3 | 4 | 5 | 4 | 4 | 4 |
| E-shock Escape Latency (Mean, sec) | X | | | 1.9 | 2.5* | 2.0 | | 2.0 | | 2.1 | 3.6**** |
| | SD | | | 0.5 | 0.7 | 0.6 | | 0.9 | | 0.7 | 0.4 |
| E-shock exposure (Total duration, sec) | X | | | 57 | 75* | 61 | 61 | 47 | 47 | 51 | 77**** |
| | SD | | | 16 | 21 | 16 | 16 | 20 | 20 | 18 | 17 |
| E-shock Distance/sec (X, Y, Z) (Mean) | X | | | 341 | 315 | 308 | 256** | 317 | 236** | 282 | 179**** |
| | SD | | | 49 | 57 | 66 | 54 | 79 | 86 | 69 | 66 |
| ITI Distance/min (X, Y, Z) (Mean) | X | 8263 | 8828 | 3996 | 4953 | 4122 | 5004** | 3978 | 5151**** | 4376 | 4961 |
| | SD | 1695 | 1258 | 1079 | 1298 | 985 | 966 | 1050 | 908 | 1018 | 907 |
| ITI Transfers (Total) | X | 56 | 58 | 20 | 30 | 27 | 26 | 20 | 30**** | 20 | 25 |
| | SD | 11 | 13 | 13 | 13 | 11 | 7 | 9 | 7 | 6 | 8 |
| ITI Rears/Climbs/Jumps (Total) | X | 50 | 50 | 18 | 21 | 24 | 27 | 13 | 25** | 17 | 23 |
| | SD | 10 | 12 | 9 | 7 | 8 | 8 | 8 | 14 | 9 | 10 |
| ITI Freezing/% Time | X | 3 | 2 | 18 | 14 | 22 | 16 | 25 | 15** | 20 | 14 |
| | SD | 2 | 1 | 10 | 7 | 12 | 6 | 10 | 8 | 9 | 7 |

$N = 17$, 17 brother pairs; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0005$.

$p < 0.0005$) (Table 2). Fig. 4E presents the e-shock escapes for ES and IS mice as 4 blocks of 6 trials each: both groups maintained a consistent frequency of escape responses across the session (Block main effect: $F < 1$, $p < 0.67$). The ES mice made a similar number of escape responses at Escape test and Pre-exposure 3 ($t = -1.33$, $p < 0.22$) and number of escape responses at Pre-exposure 3 predicted number of escape responses at Escape test ($r = 0.67$, $N = 17$, $p < 0.004$; Fig. 4F). IS mice made a similar number

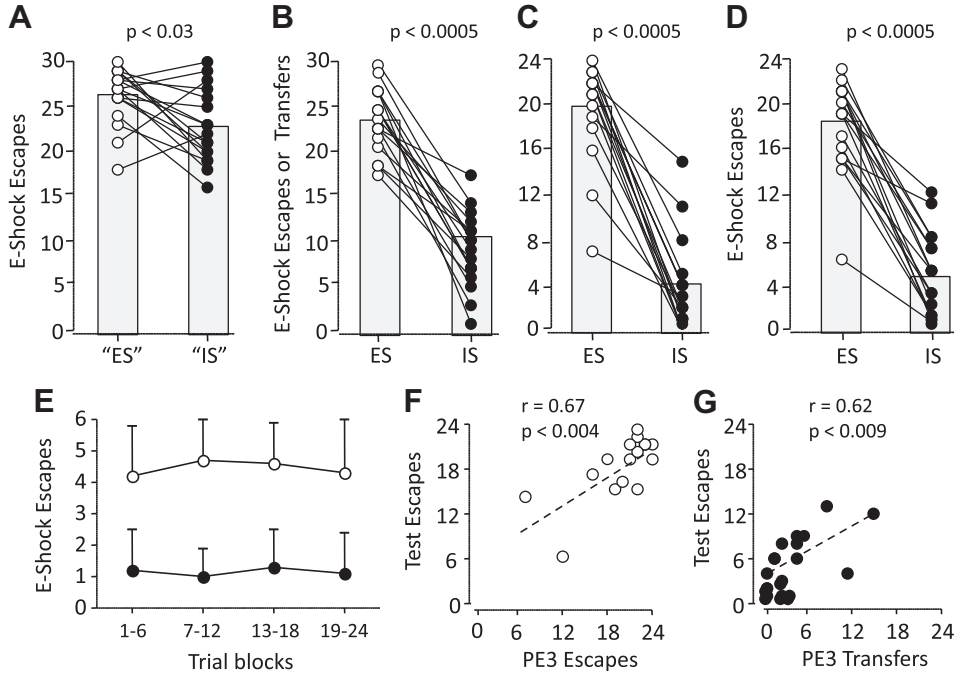



**Fig. 4.** Number of e-shock escapes or transfers by ES and IS mice (individual values for 17 brother-pairs and group means) for Experiment III, Refined conditions, Screening allocation. (A) ES Screening (Pre-exposure session 1): number of escapes by mice in response to 30 × 5-s maximum × 0.15 mA e-shocks, with 10 trials each at 75%, 50% 30% safe area in the escape compartment. In each pair, the brother with the higher mean escape latency to the last 10 e-shocks was allocated to the IS group. (B) Pre-exposure session 2: number of escapes by ES mice and transfers by IS mice in response to 30 × 5-s maximum × 0.15 mA e-shocks, with 30% safe area in the escape compartment. (C) Pre-exposure session 3: number of escapes by ES mice and transfers by IS mice in response to 24 × 4-s maximum × 0.15 mA e-shocks, with 30% safe area in the escape compartment. (D) Escape test: number of escapes by ES mice and IS mice in response to 24 × 4-s maximum × 0.15 mA e-shocks. A–D: $p$ values are for paired $t$-tests. (E) E-shock escapes (mean + SD) at Escape test, with data divided into 4 blocks of 6 trials per block to demonstrate the consistent effect of Group ($F(1, 16) = 140.75$, $p < 0.0005$) and lack of effect of Block ($F(3, 96) < 1$, $p < 0.67$) in ES and IS mice. (F) Scattergram, regression line and associated probability, and Pearson's $r$ for Pre-exposure 3 escapes versus Test escape responses in ES mice. (G) Scattergram, regression line and associated probability, and Pearson's $r$ for Pre-exposure 3 escapes versus Test escape responses in IS mice.

of escape responses at test to transfers at Pre-exposure 3 ($t = 1.11$, $p < 0.29$), and number of e-shock transfers at Pre-exposure 3 predicted number of escape responses at Escape test ($r = 0.62$, $N = 17$, $p < 0.009$; Fig. 4G).

As given in Table 2, at Pre-exposures 2 and 3, there were consistent ES–IS differences in ITI distance, with IS mice moving greater distances than ES mice and ES mice spending more time in freezing. Using the activity tracking option of the software it was possible to identify evidence for consistent ES–IS differences in the distribution of activity on the grid within the arena: as exemplified in Fig. 5, there was evidence that ES mice focussed their activity to the four corners of the grid/arena – the safe areas – during ITI's, whereas IS mice distributed their high levels of activity more equably throughout the grid/arena.

Data for pain response latencies in the hot plate test and body weight are given in Table 1. There was a significant interaction effect of Group × Time on pain response latency ($F(1, 32) = 5.03$, $p < 0.04$). *A posteriori* analysis of ES-Pre, ES-Post, IS-Pre and IS-Post did not yield a significant effect ($F(2, 32) = 3.04$, $p < 0.07$). For body weight measured on the day of Habituation (Pre) and the day of Escape test (Post), there were no significant effects of Group or Time.

It was necessary to assess whether differences in behaviour between ES and IS mice at ES Screening on Day 2 predicted, and therefore confounded, escape responses at Escape test on Day 6. The most relevant ES Screening measures were total duration of e-shock exposure on trials 1–30 and total duration of e-shock exposure on trials 21–30. Using regression, in neither the ES nor the IS group did inter-individual differences in Escape-test escape responses correlate significantly with total duration of e-shock exposure on trials 1–30 (ES: $r = 0.02$, $p < 0.96$; IS: $r = -0.28$, $p < 0.29$; Fig. 6) or total duration of e-shock exposure on trials 21–30 (ES: $r = 0.35$, $p < 0.18$; IS: $r = -0.25$, $p < 0.34$). When total duration of e-shock exposure on trials 1–30 was included as a covariate in ANCOVA with the between-subject factor of Group (ES, IS) and the dependent variable of Escape test escape responses, the covariate was not significantly related to Escape test escapes ($p < 0.44$). There was a significant main effect of Group after adjusting for the covariate scores, with Escape test e-shock escapes significantly increased in ES versus IS mice ($F(1, 31) = 69.01$, $p < 0.0005$). When total duration of e-shock exposure on trials 21–30 was included as a covariate in ANCOVA, the covariate was not significantly related to Escape test escapes ($p < 0.71$). There was a significant main effect of Group after adjusting for the covariate scores, with Escape test e-shock escapes significantly increased in ES versus IS mice ($F(1, 31) = 70.40$, $p < 0.0005$). Therefore, there was no statistical evidence that total duration of e-shock exposure (reflecting mean escape latency) of ES and/or IS mice at ES Screening predicted their escape responding at Escape test. This was in contrast to ES escape responses and IS transfer responses at Pre-exposure 3, which did correlate with escape responses at Escape test.

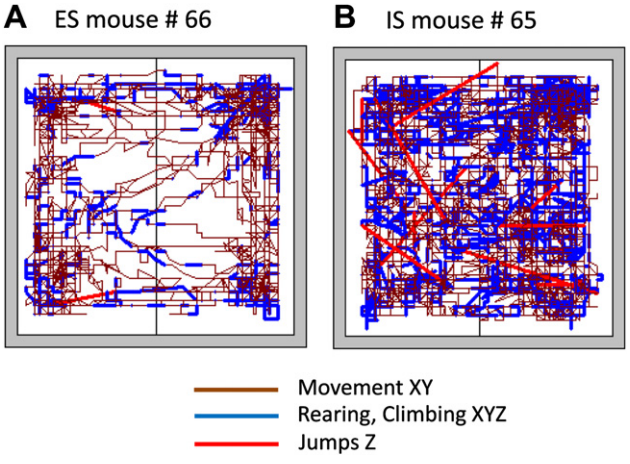



**Fig. 5.** Example of activity tracking data from Pre-exposure 3 in Experiment III, for one brother-pair, (A) ES mouse # 66 and (B) IS mouse # 65. Whereas the ES mouse focussed its relatively low levels of activity to the four "safe" corners of the grid floor and most divider-crossings were escape responses, the IS mouse distributed its relatively high levels of activity more equably throughout the grid floor area.

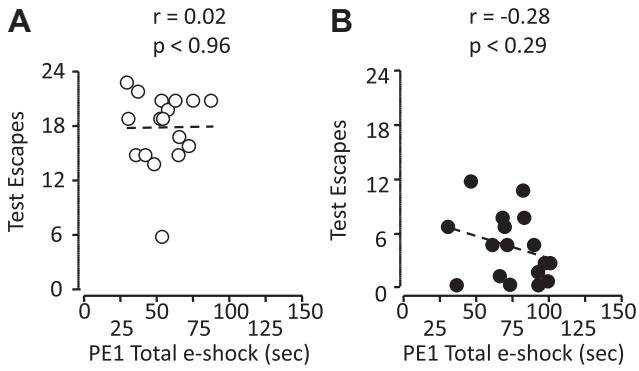



**Fig. 6.** Scattergram, regression line and associated probability, and Pearson's *r* for ES Screening (Pre-exposure 1) total e-shock exposure on trials 1–30 (proportional to mean escape latency) versus Escape test escape responses in (A) ES mice and (B) IS mice. In both groups, there was no predictive relationship between total e-shock exposure at ES Screen and escape responses at Escape test.

Given the absence of statistical evidence that inter-individual differences within or between ES and IS groups at ES Screening accounted for escape responses at Escape test, it would be predicted that in the validation experiment where brother-pairs were exposed to ES Screening but then both brothers were exposed to ES pre-exposure and Escape test, there would not be a consistent difference in Escape test escapes in mice allocated to "ES" and "IS" on the basis of their escape behaviour at ES Screening. Retrospective analysis demonstrated that there were no "ES"–"IS" behavioural differences during Habituation. At ES Screening, across trials 1–30 "ES" mice exhibited significantly more e-shock escape responses than "IS" mice ($t = 2.30$, $df = 9$, $p < 0.05$; Fig. 7A) and mean escape latency was significantly shorter in "ES" ($2.3 \pm 0.3$ s) than "IS" mice ($2.7 \pm 0.4$ s) ($t = -2$ to 29, $p < 0.05$). At PE2, PE3 and escape test, there was no significant "ES"–"IS" difference in escape responses ($p \leq 0.17$; Fig. 7B–D) and this was also the case for mean escape latency (PE1: "ES" $2.3 \pm 0.2$ s, "IS" $2.6 \pm 0.6$; PE2: "ES" $2.5 \pm 0.3$, "IS" $2.6 \pm 0.6$; Escape test: "ES" $2.6 \pm 0.4$, "IS" $2.8 \pm 0.6$, $p \leq 0.20$). Two of the ten "IS" mice exhibited notably low within-group total escape responses across all sessions (Fig. 7). At Escape test, the "ES" brothers of these two mice also exhibited low within-group total escape responses. Thus, the validation experiment provided further evidence that ES screening does not separate mice on characteristics that themselves lead to the ES–IS differences that emerge during pre-exposures and escape test. The validation experiment also provided further evidence to that observed in Experiments I and II that one brother in some pairs does develop a high level of escape failure under ES conditions.

In Experiment III main study, eleven brother-pairs were studied in tone CS – uncontrollable e-shock conditioning, using 60 pairings of tone CS that predicted an unavoidable/inescapable e-shock. During ITI's (Table 3), ES mice spent significantly more % time freezing than did IS brothers ($t = 4.07$, $df = 10$, $p < 0.003$), whereas IS mice exhibited significantly greater distance moved ($t = 3.74$, $p < 0.005$) and significantly more rears/climbs/jumps ($t = 2.76$,

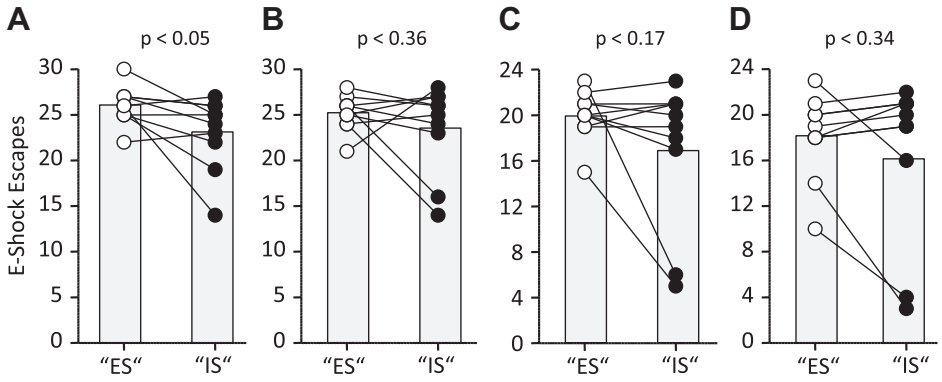



**Fig. 7.** Number of e-shock escapes or transfers by "ES" and "IS" mice (individual values for 10 brother-pairs and group means) for the Experiment III validation experiment. (A) ES Screening (Pre-exposure session 1): number of escapes by mice in response to 30 × 5-s maximum × 0.15 mA e-shocks, with 10 trials each at 75%, 50% 30% safe area in the escape compartment. In each pair, the brother with the higher mean escape latency to the last 10 e-shocks was treated as the "IS" group but received escapable e-shocks on subsequent sessions. (B) Pre-exposure session 2: number of escapes by "ES" and "IS" mice in response to 30 × 5-s maximum × 0.15 mA e-shocks, with 30% safe area in the escape compartment. (C) Pre-exposure session 3: number of escapes by "ES" and "IS" mice in response to 24 × 4-s maximum × 0.15 mA e-shocks, with 30% safe area in the escape compartment. (D) Escape test: number of escapes by "ES" and "IS" mice in response to 24 × 4-s maximum × 0.15 mA e-shocks. A–D: $p$ values are for paired $t$-tests.

$p < 0.03$) than did ES brothers. During CS's, ES mice spent significantly more % time freezing than did IS brothers ($t = 5.21$, $p < 0.0005$), and IS mice exhibited significantly greater distance moved ($t = 4.73$, $p < 0.002$) and significantly more rears/climbs/jumps ($t = 2.78$, $p < 0.03$) than did ES brothers.

To inform interpretation of these findings, a group of naive mice (NS) was given a habituation session only and then were also run in the tone CS – uncontrollable e-shock test. Data were analysed using a 1-way ANOVA (Group: ES, IS, NS). Data are presented in Table 3 and Fig. 8. During ITI's: There was a significant effect of Group on distance moved ($F(2, 27) = 7.70$, $p < 0.003$), with IS mice exhibiting significantly greater distance moved than ES mice ($p < 0.003$). There was a significant effect of Group on % time freezing ($F = 10.36$, $p < 0.0005$), with ES mice exhibiting increased freezing versus IS mice ($p < 0.0005$) and NS mice ($p < 0.04$). During CS's: There was a significant effect of Group on distance moved ($F = 13.06$, $p < 0.0005$), with IS mice exhibiting increased distance moved versus ES mice ($p < 0.0005$) and NS mice ($p < 0.002$). There was a significant effect of Group on rears/climbs/jumps ($F = 5.72$, $p < 0.01$), with IS mice exhibiting more versus ES mice ($p < 0.03$) and NS mice ($p < 0.05$). There was a significant effect of Group on % time freezing ($F = 21.50$, $p < 0.0005$), with ES mice ($p < 0.0005$) and NS mice ($p < 0.0005$) spending more time freezing than IS mice. Thus, during ITI's, ES mice maintained the relatively high level of freezing they developed during the LH procedure and IS and NS mice exhibited similar, lower levels. During CS's, ES and NS mice exhibited comparable levels of behaviour in terms of freezing, rearing/climbing/jumping and distance moved, and both groups differed significantly and similarly from IS mice. In the validation experiment, the "ES" and "IS" mice which did not exhibit a significant LH effect also did not exhibit significant differences in the CS – uncontrollable e-shock conditioning test. In both groups, behaviour was similar to that of ES mice in the main Experiment III (Table 3). For example, ITI % time freezing was 23 ± 6% in "ES" mice and 20 ± 10% in "IS" mice and CS % time freezing was 25 ± 9% in "ES" mice and 19 ± 10% in "IS" mice. These findings provide support for the interpretation that the ES–IS differences observed in main Experiment III were primarily the result of different respective ES–IS pre-exposure experiences.

**Table 3**
Data summary for tone CS – e-shock conditioning test in brother-pairs allocated to ES and IS treatment groups following screening and in a group with no e-shock prior to test (NS).

| Parameter | | ES | IS | NS |
|---|---|---|---|---|
| ITI Distance/sec (X, Y, Z) | X | 51 | 79**/** | 60 |
| | SD | 18 | 19 | 11 |
| ITI Rears/Climbs/Jumps (Total) | X | 132 | 208*/- | 115 |
| | SD | 92 | 142 | 52 |
| ITI Freezing (% Time) | X | 27 | 12**/**** | 16* vs ES |
| | SD | 11 | 7 | 3 |
| E-shock Distance/sec (X, Y, Z) | X | 221 | 183 | 229 |
| | SD | 61 | 59 | 58 |
| CS Distance/sec (X, Y, Z) | X | 51 | 79***/**** | 53*** vs IS |
| | SD | 14 | 14 | 14 |
| CS Rears/Climbs/Jumps (Total) | X | 20 | 43*/* | 21* vs IS |
| | SD | 16 | 26 | 9 |
| CS Freezing (% Time) | X | 24 | 7****/**** | 22**** vs IS |
| | SD | 8 | 5 | 6 |

$N = 11, 11, 8$ males per group for ES, IS, NS, respectively.
Total trials = 60.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0005$.
For both ES and IS, significance levels are for paired $t$-test/1-way ANOVA, respectively.

### 3.4. Experiment IV: 5-HTT knockout mice under Refined conditions, ES Screening allocation

During Habituation, there were no significant effects of Genotype (5-HTT HET, WT) or Group ("ES", "IS"). At ES Screening (Fig. 9A, D), across trials 1–24, "ES" mice exhibited significantly more e-stimulus escapes than "IS" mice (Group main effect: $F(1, 28) = 9.48$, $p < 0.005$; Fig. 9A) and mean escape latency was significantly shorter in "ES" mice than in "IS" mice (Group main effect: $F(1, 28) = 11.92$, $p < 0.002$; Table 4). ITI rears/climbs/jumps was also significantly increased in "IS" relative to "ES" mice ($F(1, 28) = 8.76$, $p < 0.006$; Table 4). ITI % time spent freezing was significantly increased in HET relative to WT mice ($F(1, 28) = 6.50$, $p < 0.02$; Fig. 9D). At Pre-exposure 2, ES mice exhibited significantly more e-shock escapes (WT–ES: 23 ± 1, HET–ES: 22 ± 2) than IS mice exhibited e-shock transfers (WT–IS: 9 ± 6, HET–IS: 7 ± 4) ($F(1, 28) = 150.22$, $p < 0.0005$). ES mice moved a greater e-shock distance than did IS mice ($F(1, 28) = 4.63$, $p < 0.04$). WT mice moved a greater ITI distance than did HET mice ($F(1, 28) = 9.20$,

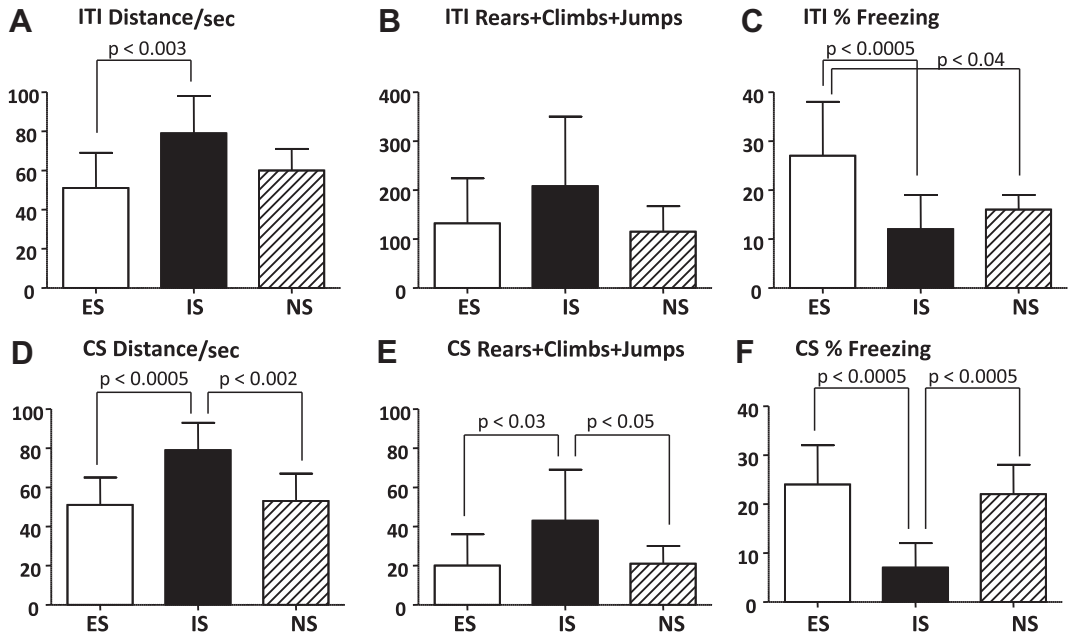



**Fig. 8.** Comparison of ES, IS and NS mouse behaviour (mean + SD, $N = 11, 11, 8$, respectively) in the tone CS-uncontrollable e-shock conditioning readout. During 59 × 50-s inter-trial intervals: (A) Mean distance moved/sec, (B) Total number of rears/climbs/jumps, (C) Mean percent time spent freezing. During 60 × 10-s CS's: (D) Mean distance moved/sec, (E) Total number of rears/climbs/jumps, (F) Mean percent time spent freezing.

$p < 0.005$). HET mice spent more % time freezing during ITI than did WT mice ($F(1, 28) = 13.36$, $p < 0.001$). At Pre-exposure 3 (Fig. 9B, E), for e-shock escapes/transfers there was a significant Genotype × Group interaction effect ($F(1, 28) = 4.66$, $p < 0.04$) and a significant main effect of Group ($F(1, 28) = 286.85$, $p < 0.0005$) (Fig. 9B, Table 4). *A posteriori* one-way ANOVA and *post hoc* testing demonstrated that: HET–IS mice performed significantly less transfers than did WT–IS ($p < 0.007$); HET–IS mice performed

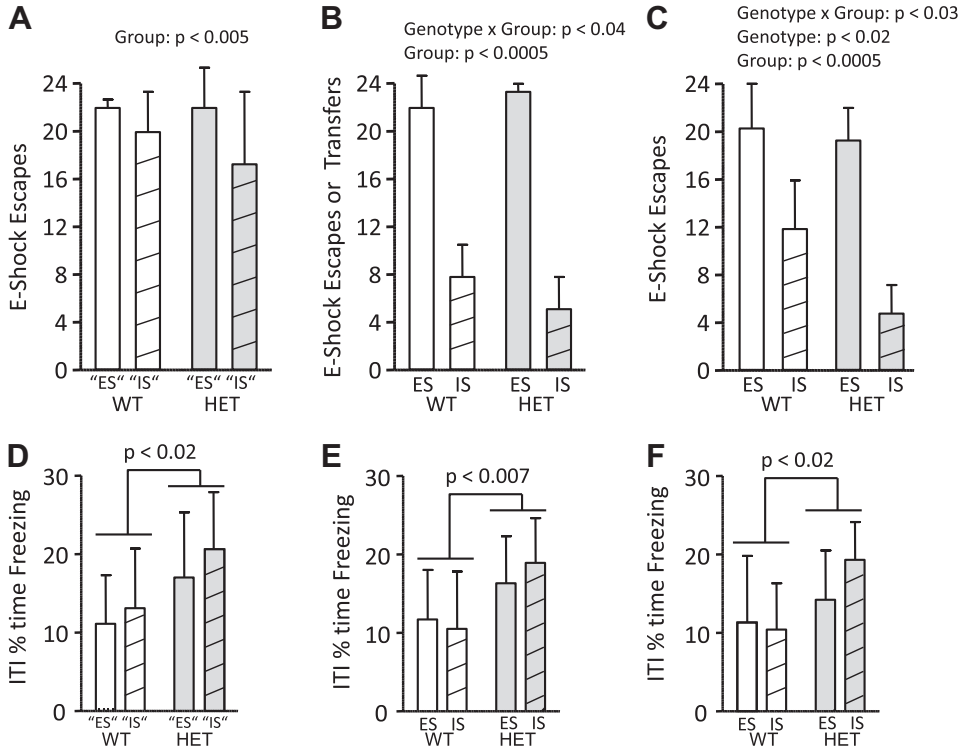



**Fig. 9.** Number of e-shock escapes or transfers and % ITI time spent freezing by ES and IS mice of WT or HET genotype in the 5-HTT knockout strain (mean ± SD for 8 brother-pairs per genotype) for Experiment IV, Refined conditions, Screening allocation. (A) ES Screening (Pre-exposure session 1): number of escapes by mice in response to 24 × 5-s maximum × 0.15 mA e-shocks, with 8 trials each at 75%, 50% 30% safe area in the escape compartment. In each pair, the brother with the higher mean escape latency to the last 8 e-shocks was allocated to the IS group. (B) Pre-exposure session 3: number of escapes by ES mice and transfers by IS mice in response to 24 × 4-s maximum × 0.15 mA e-shocks, with 30% safe area in the escape compartment. (C) Escape test: number of escapes by ES mice and IS mice in response to 24 × 4-s maximum × 0.15 mA e-shocks. (D) ES Screening: % ITI spent freezing. (E) Pre-exposure session 3: % ITI spent freezing. (F) Escape test: % ITI spent freezing. A–F: *p* values are for factorial ANOVA.

**Table 4**
Summary table for experiment with 5-HTT HET–HET or WT–WT brother-pairs allocated to ES and IS treatment groups following screening.

| Parameter | | Pre-exposure 1 | | | | Pre-exposure 3 | | | | Escape test | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | WT | WT | HET | HET | WT | WT | HET | HET | WT | WT | HET | HET |
| | | ES | IS | ES | IS | ES | IS | ES | IS | ES | IS | ES | IS |
| E-shock, ES Escapes/IS Transfers (Total) | X | 23 | 20 | 22 | 17** | 22 | 8 | 23 | 5§, **** | 20 | 11 | 19 | 4§, ****, # |
| | SD | 1 | 4 | 4 | 6 | 3 | 3 | 1 | 3 | 4 | 5 | 3 | 3 |
| E-shock Escape Latency (Mean, sec) | X | 2.1 | 2.9 | 2.3 | 3.3** | 2.7 | 2.3 | | | 2.8 | 3.6 | 2.6 | 3.8**** |
| | SD | 0.4 | 0.6 | 0.8 | 1.1 | 1.0 | | 0.6 | | 1.0 | 0.5 | 0.7 | 0.4 |
| E-shock exposure (Total duration, sec) | X | 50 | 71 | 55 | 80** | 68 | | 59 | | 66 | 86 | 63 | 92**** |
| | SD | 9 | 13 | 20 | 27 | 23 | | 14 | | 24 | 11 | 17 | 10 |
| E-shock Distance/sec (X, Y, Z) (Mean) | X | 314 | 286 | 319 | 289 | 266 | 204 | 298 | 199** | 248 | 187 | 249 | 143*** |
| | SD | 28 | 54 | 34 | 58 | 81 | 50 | 87 | 95 | 58 | 51 | 81 | 48 |
| ITI Distance/min (X, Y, Z) (Mean) | X | 4054 | 4852 | 3878 | 3885 | 5181 | 5519 | 4646 | 3859# | 6047 | 4914 | 4985 | 4026*, # |
| | SD | 1049 | 1501 | 1150 | 870 | 1722 | 2049 | 1029 | 745 | 1646 | 1177 | 1195 | 1122 |
| ITI Transfers (Total) | X | 11 | 18 | 11 | 12 | 25 | 32 | 28 | 23 | 30 | 26 | 26 | 24 |
| | SD | 8 | 14 | 9 | 10 | 18 | 16 | 5 | 7 | 9 | 18 | 7 | 7 |
| ITI Rears/Climbs/Jumps (Total) | X | 25 | 67 | 28 | 41** | 34 | 35 | 20 | 28# | 39 | 59 | 38 | 53 |
| | SD | 14 | 40 | 21 | 24 | 15 | 16 | 10 | 14 | 21 | 51 | 24 | 66 |
| ITI Freezing/% Time | X | 11 | 13 | 17 | 21# | 12 | 11 | 16 | 19## | 11 | 10 | 14 | 19# |
| | SD | 6 | 8 | 8 | 7 | 6 | 7 | 6 | 6 | 9 | 6 | 6 | 5 |

$N = 8, 8, 8, 8$ males per group for WT–ES, WT–IS, HET–ES, HET–IS; * ES vs IS, # HET vs WT, § (ES vs IS) × (HET vs WT); *#§ $p < 0.05$, **## $p < 0.01$, *** $p < 0.001$, **** $p < 0.0005$.

significantly less transfers than HET–ES ($p < 0.0005$) and WT–ES ($p < 0.0005$) mice performed escapes; and WT–IS mice performed significantly less transfers than WT–ES ($p < 0.0005$) and HET–ES ($p < 0.0005$) mice performed escapes. For e-shock distance moved there was a significant main effect of Group ($F(1, 28) = 8.00$, $p < 0.009$; Table 4), with IS mice moving a shorter distance than ES mice. For ITI distance moved there was a significant main effect of Genotype ($F(1, 28) = 4.39$, $p < 0.05$; Table 4), with HET mice moving a shorter distance than WT mice. For ITI % time spent freezing, there was a significant main effect of Genotype ($F(1, 28) = 8.57$, $p < 0.007$; Fig. 9E), with HET mice spending more time freezing than WT mice. At Escape test (Fig. 9C, F), for e-shock escapes there was a significant Genotype × Group interaction effect ($F(1, 28) = 6.03$, $p < 0.03$) and significant main effects of Genotype ($F(1, 28) = 6.51$, $p < 0.02$) and Group ($F(1, 28) = 80.53$, $p < 0.0005$; Fig. 9C). *A posteriori* one-way ANOVA and *post hoc* testing demonstrated that: HET–IS mice performed significantly less escapes than HET–ES ($p < 0.0005$), WT–ES ($p < 0.0005$) and WT–IS ($p < 0.009$) mice; and WT–IS mice performed significantly less escapes than WT–ES ($p < 0.0005$) and HET–ES ($p < 0.001$) mice. For mean e-shock escape latency there was a significant main effect of Group ($F(1, 28) = 17.91$, $p < 0.0005$; Table 4) with IS mice exhibiting a significantly longer latency. For e-shock distance moved there was a significant main effect of Group ($F(1, 28) = 15.20$, $p < 0.001$; Table 4) with IS mice moving a shorter distance. For ITI distance moved there were significant main effects of Genotype ($F(1, 28) = 4.49$, $p < 0.05$) and Group ($F(1, 28) = 5.16$, $p < 0.04$), with HET moving a shorter distance than WT mice and IS moving a shorter distance than ES mice (Table 4). For ITI % time spent freezing, there was a significant main effect of Genotype ($F(1, 28) = 6.60$, $p < 0.02$; Fig. 9F) with HET mice spending more time freezing than WT mice.

Data for pain response latencies in the hot plate test and body weight are given in Table 1. For pain response latency ($p \geq 0.10$) and body weight ($p \geq 0.37$) there were no significant effects of Genotype, Group or Time.

## 4. Discussion

Objective mouse behaviour analogues of human emotional, motivational and cognitive processes underlying psychiatric disorders are essential to valid translational pre-clinical research into the aetiology and pathophysiology of those disorders and to predictive neuropsychopharmacological screening of potential novel treatments. Conducting mouse behavioural studies with tests of negligible face and construct validity is a major bottle-neck to progress, as no amount of development of molecular-genetic tools for the manipulation of the mouse brain and imaging tools for the visualization of its activity will bring progress unless they can be combined with psychopathology models of high aetiological, face and construct validity (Krishnan and Nestler, 2008; Markou et al., 2009; Willner and Mitchell, 2002). Against this background, the present development of a robust mouse paradigm of the LH effect is a significant step forward, particularly with respect to objective face validity.

The starting point of the study (Experiment I) was to use a standard two-way shuttle box, as has been used previously in mice to study the LH (IS–ES) effect or the aversive US pre-exposure/interference (IS–NS) effect (Anisman and Merali, 2001; Randich and LoLordo, 1979). A standard open gate delineating a single, immediate transfer point from the e-shock- to the safe-compartment, was used. Also as is typical, mice were allocated randomly to the ES and IS groups (Anisman and Merali, 2001). Brother-pairs were used because this: allowed subjects to be caged socially until 1 week prior to the experiment without the risk of high aggression; could reduce the extent of any pre-existing individual differences ((epi)genetic, early environmental) between mice allocated randomly to the same ES–IS dyad; and was consistent with the dependent-pair statistical model used for analysis. Under these standard conditions, it was possible to demonstrate a borderline non-significant deficit in number of escape responses by IS relative to ES mice at test. This weak LH

effect is in broad agreement with previous studies using C57BL/6 mice and standard conditions. For example, one aversive US pre-exposure effect (IS–NS) study reports that only 30% of mice exposed to 360 IS e-shocks of 1–3 s × 0.15 mA subsequently failed to escape the majority of 10-s escapable e-shocks at test; the remaining 70% of IS mice exhibited few escape failures and the average escape latency of 2.5 s at test was similar to that of the ES mice in the present experiment (Chourbaji et al., 2005). A study in Swiss-Webster mice that used a similar design to that used here, albeit with a closable gate and 6-s delay between e-shock onset and gate opening, and a 24-s maximum e-shock duration, observed a robust LH effect (Anisman et al., 1978). In the present experiment I, IS mice made more escape responses at test than e-stimulus transfers at final IS pre-exposure, and in terms of inter-individual differences the latter predicted the former. At the final pre-exposure session, there was an average probability of 0.29 (range 0.0–0.67) that an IS mouse would transfer through the gate, even though this behaviour had not been reinforced across three pre-exposure sessions. These findings suggest a superstitious but nonetheless pre-adaptive association between gate transfer and e-shock termination, such that the habit of IS gate transferring was sufficient to support within-session escape learning by approximately half of the IS mice during the escape test. This development of spontaneous gate transfer during IS pre-exposure made a substantial contribution to there being only a marginal LH effect. The other contributing factor was that two ES mice developed low levels of escape responding, suggesting that repeated experience of two-way e-shocks, although escapable, nonetheless induced a state of uncontrollability in these mice. The brothers of these two mice exhibited considerably more escape responses at test despite having experienced only IS – and at relatively long durations – previously. The marked within-group ES and IS differences suggest that mice exhibit marked individual differences in both ability to utilise controllability of an aversive situation (i.e. ES) and in their vulnerability-resilience to the uncontrollability of an aversive situation (i.e. IS). That ES mice that were poor escapers had IS brothers that were relatively good escapers, suggests that within-litter (intra-familial) differences can contribute substantially to inter-individual differences.

The aim of experiment II was to investigate whether (i) replacement of the gate with a divider that allowed movement between compartments across the depth of the arena, combined with (ii) gradual separation of the central divider from the border of the safe area of the opposite compartment, would result in a reduction in the spontaneous transfers during IS pre-exposure that appeared to act as a superstitious pre-adaptation to escape learning, and thereby lead to a robust LH effect. At the final pre-exposure session the average probability that an IS subject would transfer under the divider to the safe area was halved relative to experiment I, to 0.17 (range 0.00–0.67) and, probably as a consequence of this low transfer rate, there was no overall increase in escape responses at test relative to transfers at final pre-exposure in IS mice. This led to a significant LH effect being obtained under these test conditions. Apparently therefore, a threshold rate of pre-adaptive spontaneous transfer is required to support escape learning at test In IS mice. These findings provide an interesting parallel to the findings of the rat LH model in which an LH effect is observed when two crossings of the shuttle arena are required for escape (fixed ratio 2, FR2) but not at FR1 (e.g. Jackson et al., 1978). Taken together, the mouse and rat findings suggest that the escape response must pose a certain level of cognitive challenge to ES and IS subjects for a consistent LH effect to be obtained. Although the LH effect was statistically significant, there was marked within-group variability and therefore some between-group overlap in escape test escape responses. As in Experiment I, a minority of ES mice – in this case 3 of 12 – developed low levels of escape responding across repeated ES sessions. Furthermore, the IS brothers of these mice exhibited high levels of escape at test, in both relative and absolute terms. This suggests that in some mice, the experience of IS followed by ES leads to a highly reinforcing effect of ES that could be related to the emotion of relief. This phenomenon will be the subject of future studies in this paradigm.

The aim of experiment III was to investigate whether exposure of both males of each brother-pair to a session of escapable e-shocks would (i) allow for screening of individual differences between brothers in their ability to exhibit behavioural control over e-shocks in the modified two-way escape set-up, such that (ii) assignment of the brother exhibiting the shorter mean escape latency at the end of the screening session to the ES group would reduce subsequent within-group variability in both ES and IS mice and thereby increase the robustness of the LH effect. The differences within brother-pairs in number of escape responses and average escape latency across the entire screening session were typically small. However, they did increase towards the end of the session, and assigning the brother with the shorter mean escape latency in the final 10 trials of screening to the ES group resulted in each ES male exhibiting more escapes at test than did its IS brother and therefore a robust LH effect. Screening results in an IS group that has experienced more total e-shock exposure prior to the escape test and this could be a confounding variable. To demonstrate that the subsequent ES–IS pre-exposure sessions and not the screening procedure was the primary factor underlying the LH effect, several validation steps were carried out. These included statistical demonstrations that duration of e-shock exposure/latency of escape responses at screening did not predict escape responses in ES or IS mice whereas e-shock exposure at final pre-exposure did; e-shock exposure at screening was not a significant covariate of escape responses at test and the ES–IS effect on escape responses at test remained significant after adjusting for the covariate; a separate experiment demonstrating that screening followed by ES only did not lead to an LH effect. Nonetheless, it is clear from Experiments I and II that there are brother-pairs within which there are marked pre-existing differences that contribute to their relative escape behaviour at escape test, in addition to/interaction with the effects of ES and IS. The cause of these pre-existing within-family differences are of course highly relevant (see Experiment IV). Indeed, inadequate consideration of individual differences in vulnerability-resilience to helplessness following uncontrollable life events has been a major criticism of the LH theory of human depression (Miller and Norman, 1979). One possibility is that high differences in social status and agonistic interactions within brother-pairs were a contributing factor to development of behavioural differences in the LH effect paradigm. Certainly, inter-male aggression is more marked in mice than rats, and this could lead to more marked differences in capacity for controllability in mice, such that random allocation to ES and IS groups is more problematic than it is in rats (Amat et al., 2005; Maier and Seligman, 1976). Finally here, it is important to note that ES screening could have resulted in immunization of the IS mice against the effects of subsequent IS. Immunization i.e. exposure of an IS group to ES prior to IS, has been demonstrated to block the LH effect in rat (e.g. Amat et al., 2006) and mouse (Anisman et al., 1983). In the mouse study, mice were exposed to escapable e-shock or no e-shock in a shuttle arena (day 1), inescapable e-shock or no e-shock in a single compartment arena (day 2), and escape test in the shuttle arena (day 3). Mice that received escapable e-shock on day 1 and inescapable e-shock on day 2 did not exhibit an escape deficit on day 3 relative to mice that received no e-shock on day 2, i.e. no LH effect (Anisman et al., 1983). One major difference of this study to the current study was that in the latter

mice experienced ES, IS and escape test in the same environment, and it could be that an immunization effect of ES was blocked by the subsequent association of IS with the escape test context. Clearly, however, it cannot be assumed that there was no immunization effect in the present study.

Also in experiment III, evidence was obtained in the ES–IS pre-exposure sessions, escape test and CS-uncontrollable e-shock test, that the controllability status of e-shock is associated with the development of differences in its emotional valence for ES versus IS mice. Firstly, relative to ES mice, IS mice exhibited reduced motor responses to e-shock (e-shock distance/sec) during pre-exposure sessions and escape test. The causality of the reduced motor response to e-shock and the causal contribution of this reduced motor response *per se* to the LH effect, are currently unclear. In earlier studies, which did not accurately quantify motor responses, the LH effect in mouse was attributed to a decline in motor response initiation and maintenance following exposure to IS specifically (Anisman et al., 1978), and IS mouse-specific decreases in central noradrenaline and dopamine were proposed to underlie this (Anisman et al., 1979, 1980). These earlier studies did not have the benefit of being able to accurately quantify motor responses, and the current data may provide the first quantitative evidence for reduced motor response to e-shock in IS mice. However, the present data also indicate that IS mice exhibited increased ITI motor activity relative to ES mice, and this effect cannot be readily reconciled with a motor-deficit explanation for the LH effect. Alternatively, it is possible that the reduced motor response to e-shock, specifically, reflects reduced incentive-motivation to attempt to escape in the IS mice, in accordance with the motivational component of the original interpretation of the LH effect (Maier and Seligman, 1976). This explanation would also infer a major role for monoamine neurotransmission, but in terms of reward/punishment-goal-action pathways, rather than motor activity *per se* (Huys and Dayan, 2009). A further difference in ES–IS behaviour was the relatively high amount of ITI time spent in freezing by ES relative to IS mice, both during Pre-exposure session 3 and CS conditioning test. Direct anecdotal observation, combined with freezing scores and activity tracking images, indicated that ES mice spent a relatively high proportion of the ITI's between escapable e-shocks in freezing in one or other of the corners of the arena, and facing towards the opposite arena side. As such this ITI freezing could have reflected a state of readiness to escape the e-shock. ES mice maintained this relatively high level of freezing behaviour during the CS conditioning test; here the state-of-readiness explanation would not apply as IS conditions were applied, so that freezing might have carried over as a habit from ES sessions. Certainly, NS mice also exhibited a lower level of ITI freezing than did ES mice during the CS conditioning test, indicating that high ITI freezing was specific to the ES mice. During CS's that predicted IS's, freezing behaviour was exhibited to a similar level in ES and NS mice, and to a relatively high level in both groups compared to IS mice. The latter exhibited increased CS distance moved and CS rears/climbs/jumps relative to both ES and NS mice that again exhibited similar levels of these behaviours to each other. Integrating these findings, we propose that: (i) High ITI freezing in ES mice reflects development of a controlled adaptive anticipatory response to an escapable aversive stimulus in ES sessions, which is maintained as a habit when the stimulus becomes inescapable. (ii) High CS-induced freezing – as typically observed in fear conditioning experiments – by ES and NS mice constitutes a controlled, moderate response to a signalled uncontrollable aversive stimulus. (iii) High CS horizontal-vertical motor activity – as rarely observed in fear conditioning experiments – by IS mice constitutes an uncontrolled, marked response to a signalled uncontrollable aversive stimulus. That is, chronic exposure to an uncontrollable aversive stimulus leads to development of a potentiated uncontrolled response (e.g. panic-flight (Blanchard et al., 2001)) to stimuli that accurately predict the imminence of that uncontrollable stimulus. The preceding interpretation is clearly speculative and requires further investigation. Furthermore, it is important to note that in rat LH studies it has been demonstrated that freezing to context is increased in IS relative to ES subjects (e.g. Grahn et al., 2000), which is the opposite of the current findings. One possible explanation is that the amplitude of e-shock used in the present study was below that required to yield fear-conditioned freezing to context but does support fear-conditioned freezing to a discrete, predictive CS. This explanation would fit with the low ITI freezing by IS mice during the LH procedure, the adaptive state-of-readiness explanation put forward to account for relatively high ITI freezing by ES mice during the LH procedure, the controlled response explanation put forward to account for relatively high CS freezing by ES and NS mice, and the uncontrolled response explanation put forward to account for relatively high CS vertical-horizontal motor activity by IS mice. When ES and IS rats are compared on fear-conditioned freezing to CS, IS potentiates fear-induced freezing to the CS (Barratta et al., 2007). According to our proposed interpretation of the high horizontal-vertical motor activity induced by the CS in IS mice, IS would potentiate CS fear conditioning in both rats and mice. Finally here, as for the LH effect, the validation experiment provided evidence that the ES–IS differences observed in the CS conditioning test were due to ES–IS pre-exposure and were not confounded by ES screening.

The aim of Experiment IV was to apply the LH effect model to study the effects of constitutional heterozygote knockout of the serotonin transporter gene. Genetically-determined reduced 5-HTT function is an aetiological risk factor for depression in interaction with aversive life events (Caspi et al., 2003; Murphy and Lesch, 2008). If the LH effect model has validity for depression-relevant neurobiological and –pharmacological studies, it would be expected to demonstrate an increased LH effect in HET relative to WT mice. The ES screening design was used as in Experiment III. In this 5-HTT ko strain on a C57BL/6 background, a robust LH effect was observed similar to that obtained with C57BL/6 mice in Experiment III. This demonstrates that the model has applicability to other strains, although a pilot study is recommended to optimise parameters for each strain e.g. the number of trials per session, which was reduced in this 5-HTT ko strain experiment relative to the C57BL/6 experiments. Furthermore, the LH effect was more marked in the HET mice relative to WT and this was exclusively due to the low number of escapes by HET–IS mice relative to WT–IS mice. This finding indicates that developing with only one copy of the 5-HTT gene increases sensitivity to uncontrollable e-shock in terms of increased escape failure at escape test. Indeed, the HET–IS mice also exhibited reduced transfers relative to their WT–IS counterparts in the final pre-exposure session. It has recently been demonstrated that 5-HTT HET mice exhibit increased escape failure relative to WT following IS (Muller et al., 2011), and the present study replicates this finding. However, the previous study was conducted without an ES comparison group (i.e. the US pre-exposure effect paradigm), so it was unclear whether the phenotype was at all related to the uncontrollability of the e-shock. Using the LH model it has been possible to demonstrate that the 5-HTT HET phenotype is indeed entirely due to uncontrollability. The neuropsychological processes underlying this genotype $\times$ environment interaction ($G \times E$) require and warrant detailed investigation, but there are some interesting co-findings in the current experiment that could well be relevant. Particularly interesting is the increased time spent in freezing behaviour by HET mice relative to WT mice and in both ES

and IS groups. This increased freezing was already present at ES screening – but not at habituation – and was maintained up to and including escape test. This suggests that HET mice exhibit an endophenotype of increased fear conditioning to context associated with aversive stimuli and that this endophenotype contributes to development of LH if uncontrollability is experienced in such environments. There are interesting parallels with the human situation, given that healthy individuals with the *short* variant of the regulatory gene-linked polymorphic region of the 5-HTT gene (reduced 5-HTT function, cf. 5-HTT HET mouse) exhibit increased reactivity to fearful stimuli (Phelps and LeDoux, 2005) and a relatively high prevalence of depression when exposed to several aversive life events (Caspi et al., 2003; Karg et al., 2011). The important inference that can be made from the present experiment is that uncontrollability in these life events is a key factor in their aetio-pathology.

With respect to the important question of which is the optimal experimental design for the study of the LH effect, it would appear that this is dependent on the research question. This situation is illustrated in Fig. 10. In studies where the aim is to investigate the neurobiology of the LH effect, then random allocation is superior because this would maximise the extent to which ES–IS differences are attributable to differential experience of the un/controllability of the aversive stimulus *per se*. To eliminate brother-pairs where one or two brothers exhibit an "escape failure trait" i.e. vulnerability to develop escape failure even if exposed to ES, minimum thresholds can be set for performance at Pre-exposure 1. In studies where the aim is to investigate the contribution of genetic factors to the LH effect, as in Experiment IV, then ES screening is superior because this ensures that the different genotype groups are balanced with respect to allocation of mice to ES and IS. It also ensures that mice with an "escape failure trait" are allocated to the IS group and allows this to be compared with the equivalent WT–IS group. In pharmacological studies, both designs are appropriate. For example, it would be desirable to include mice with an "escape failure trait" in a study of a putative anti-depressant compound because such mice would constitute the at-risk-for-LH population.

Establishment of a mouse model to demonstrate robust effects of the uncontrollability *per se* of a specific, chronically aversive environment provides a significant advance in the methods available for valid translational study of psychological processes of major relevance to depression. In accordance with the original LH effect theory (Maier and Seligman, 1976), there is evidence that the impact of the aversive uncontrollability was: emotional, evidenced by uncontrolled responding to CS's that predicted uncontrollable e-shocks; incentive-motivational, evidenced by reduced motor responding to e-shocks; cognitive, evidenced by a consistent low level of escape responses across escape test without development of response-outcome association/expectancy. The parsimonious view is that this constitutes a specific learned aversive uncontrollability (LAU) effect and not a generalized LH effect. The LAU effect was achieved with a duration and intensity of e-shocks that were minimal relative to those typically used – in this respect the model represents significant refinement and reduction with respect to the 3R's of animal welfare. Withstanding the qualification that the effect is specific and does not itself constitute LH, this model is important given that it will enable the study of: (i) The contributions of emotional, motivational and cognitive processes, and their interaction, to the LAU effect. (ii) The generalized LH effect: one of the major applications will be to investigate whether prior experience of uncontrollable aversive events other than e-shocks, e.g. chronic social defeat (Krishnan and Nestler, 2008) induces a spontaneous escape deficit in the current escape test, which would constitute a model of a generalized LH effect, given that such mice would not have prior experience of e-shocks as uncontrollable. That is, the current deficit in IS mice provides a comparator with which two-way escape deficits exhibited by mice exposed to other aversive environments can be interpreted as being underlain by the

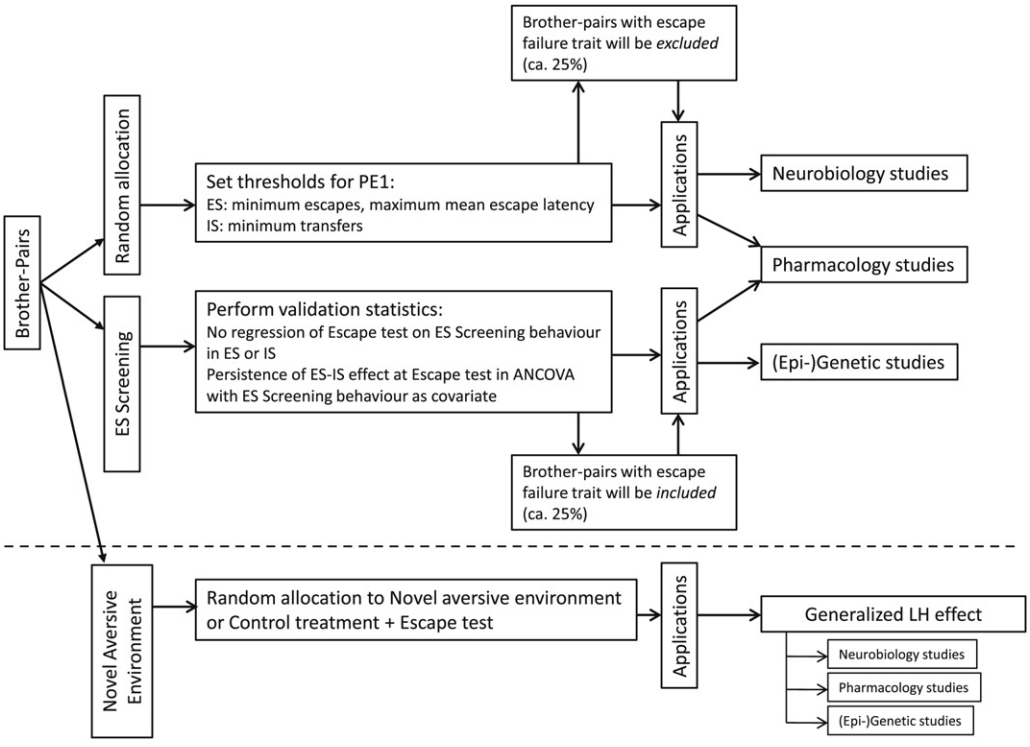



**Fig. 10.** Flow diagram depicting the relationship between appropriate experimental design and research question for the mouse LH effect paradigm.

psychological state of (generalized) uncontrollability (Fig. 10). (iii) The neural circuitry and synaptic neurobiology of the LAU and LH effects. This will include the application of molecular-genetic tools to study the contribution of specific genetic and gene–environment interaction effects to the LAU effect and LH effect, as conducted here for the 5-HTT gene. (iv) Discovery and validation of novel therapeutic targets, and the screening of compounds and biologics, for reversal of the LH effect. As such, this establishment of a mouse model of the classical translational paradigm of learned aversive uncontrollability will provide extensive opportunities for psychopathological research in depression with aetiological, face and construct validity, with potential for delivery of pre-clinical evidence that will translate into improved understanding and treatment of human depression.

## Disclosures

The authors have no disclosures.

## Acknowledgements

Research was funded by the Swiss National Science Foundation (grant 31003A_130499) and the National Center for Competence in Research “Neural Plasticity and Repair”. We thank Daniel Schuppli and his team for animal care. We thank TSE Systems GmbH for the opportunity to co-develop the Multiconditioning System hardware and software to enable the current study to be conducted.

## References

Abramson, L.Y., Metalsky, G.I., Alloy, L.B., 1989. Hopelessness depression: a theory-based subtype of depression. Psychol. Rev. 96, 358–372.
Abramson, L.Y., Seligman, M.E.P., Teasdale, J.D., 1978. Learned helplessness in humans: critique and reformulation. J. Abnorm. Psychol. 87, 49–74.
Alloy, L.B., Abramson, L.Y., Whitehouse, W.G., Hogan, M.E., Tashman, N.A., Steinberg, D.L., Rose, D.T., Donovan, P., 1999. Depressogenic cognitive styles: predictive validity, information processing and personality characteristics, and developmental origins. Behav. Res. Ther. 37, 503–531.
Amat, J., Baratta, M.V., Paul, E., Bland, S.T., Watkins, L.R., Maier, S.F., 2005. Medial prefrontal cortex determines how stressor controllability affects behavior and dorsal raphe nucleus. Nat. Neurosci. 8, 365–371.
Amat, J., Paul, E., Zarza, C., Watkins, L.R., Maier, S.F., 2006. Previous experience with behavioral control over stress blocks the behavioral and dorsal raphe nucleus activating effects of later uncontrollable stress: role of the ventral medial prefrontal cortex. J. Neurosci. 20, 13264–13272.
Anisman, H., deCatanzaro, D., Remington, G., 1978. Escape performance following exposure to inescapable shock: deficits in motor response maintenance. J. Exp. Psychol. Anim. Behav. Proc. 4, 197–218.
Anisman, H., Irwin, J., Sklar, L.S., 1979. Deficits of escape performance following catecholamine depletion: implications for behavioral deficits induced by uncontrollable stress. Psychopharmacology 64, 163–170.
Anisman, H., Irwin, J., Beauchamp, C., Zacharko, R., 1983. Cross-stressor immunization against the behavioral deficits introduced by uncontrollable shock. Behav. Neurosci. 97, 452–461.
Anisman, H., Merali, Z., 2001. Rodent models of depression: learned helplessness induced in mice. Curr. Protoc. Neurosci. Behavioral Neuroscience (Suppl. 14) 810C.811–810C.815.
Anisman, H., Pizzino, A., Sklar, L.S., 1980. Coping with stress, norepinephrine depletion and escape performance. Brain Res. 191, 583–588.
Barratta, M.V., Christianson, J.P., Gomez, D.M., Zarza, C.M., Amat, J., Masini, C.V., Watkins, L.R., Maier, S.F., 2007. Controllable versus uncontrollable stressors bi-directionally modulate conditioned but not innate fear. Neuroscience 146, 1495–1503.
Bengel, D., Murphy, D.L., Andrews, A.M., Wichems, C.H., Feltner, D., Heils, A., Mössner, R., Westphal, H., Lesch, K.-P., 1998. Altered brain serotonin homeostasis and locomotor insensitivity to 3,4-methylenedioxymethamphetamine (“ecstasy”) in serotonin transporter-deficint mice. Mol. Pharmacol. 53, 649–655.
Blanchard, D.C., Griebel, G., Blanchard, R.J., 2001. Mouse defensive behaviors: pharmacological and behavioral assays for anxiety and panic. Neurosci. Biobehav. Rev. 25, 205–218.
Caspi, A., Sugden, K., Moffitt, T.E., Taylor, A., Craig, I.W., Harrington, H., McClay, J., Mill, J., Martin, J., Braithwait, A., Poulton, R., 2003. Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science 301, 386–389.
Castagné, V., Moser, P., Roux, S., Porsolt, R.D., 2011. Rodent models of depression: forced swim and tail suspension behavioral despair tests in rats and mice. Curr. Protoc. Neurosci. Chapter 8: Unit 8.10A.
Chang, T., Meyer, U., Feldon, J., Yee, B.K., 2007. Disruption of the US pre-exposure effect and latent inhibition in a two-way active avoidance by systemic amphetamine in C57BL/6 mice. Psychopharmacol 191, 211–221.
Chourbaji, S., Zacher, C., Sanchis-Segura, C., Dormann, C., Vollmayr, B., Gass, P., 2005. Learned helplessness: validity and reliability of depressive-like states in mice. Brain Res. Protoc. 16, 70–78.
DSM-IV, 1994. Diagnostic and Statistical Manual of Mental Disorders, fourth ed. American Psychiatric Association, Washington, DC.
El Yacoubi, M., Popa, D., Martin, B., Zimmer, L., Hamon, M., Adrien, J., Vaugeois, J.-M., Genetic association between helpless trait and depression-related phenotypes: evidence from crossbreeding studies with H/Rouen and NH/Rouen mice. Int. J. Neuropsychopharmacol, in press. doi:10.1017/S1461145711000605.
Grahn, R.E., Watkins, L.R., Maier, S.F., 2000. Impaired escape performance and enhanced conditioned fear in rats following exposure to an uncontrollable stressor are mediated by glutamate and nitric oxide in the dorsal raphe nucleus. Behav. Brain Res. 112, 33–41.
Harrow, M., Hansford, B.G., Astrachan-Fletcher, E.B., 2009. Locus of control: relation to schizophrenia, to recovery, and to depression and psychosis – 1 15-year longitudinal study. Psychiatry Res. 168, 186–192.
Huys, Q.J.M., Dayan, P., 2009. A Bayesian formulation of behavioral control. Cognition 113, 314–328.
ICD-10, 1994. International Statistical Classification of Diseases and Related Health Problems 10th Revision.
Jackson, R.L., Maier, S.F., Rapaport, P.M., 1978. Exposure to inescapable shock produces both activity and associative deficits in the rat. Learn. Motiv. 9, 69–98.
Karg, K., Burmeister, M., Shedden, K., Sen, S., 2011. The serotonin transporter promoter variant (5-HTTLPR), stress, and depression meta-analysis revisited. Arch. Gen. Psychiatry. 68, 444–454.
Keck, M.E., Welt, T., Müller, M.B., Uhr, M., Ohl, F., Wigger, A., Toschi, N., Holsboer, F., Landgraf, R., 2003. Reduction of hypothalamic vasopressinergic hyperdrive contributes to clinically relevant behavioral and neuroendocrine effects of chronic paroxetine treatment in a psychopathological rat model. Neuropsychopharmacol 28, 235–243.
Krishnan, V., Nestler, E.J., 2008. The molecular neurobiology of depression. Nature 455, 894–902.
Lu, A., Steiner, M.A., Whittle, N., Vogl, A.M., Walser, S.M., Ableitner, M., Refojo, D., Ekker, M., Rubenstein, J.L., Stalla, G.K., Singewald, N., Holsboer, F., Wotjak, C.T., Wurst, W., Deussing, J.M., 2008. Conditional mouse mutants highlight mechanisms of corticotropin-releasing hormone effects on stress-coping behavior. Mol. Psychiatry 13, 1028–1042.
Maeng, S., Zarate, C.A., Du, J., Schloesser, R.J., McCammon, J., Chen, G., Manji, H.K., 2008. Cellular mechanisms underlying the antidepressant effects of ketamine: role of a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors. Biol. Psychiatry 63, 349–352.
Maier, S.F., Seligman, M.E.P., 1976. Learned helplessness: theory and evidence. J. Exp. Psychol. Gen. 105, 3–46.
Markou, A., Chiamulera, C., Geyer, M.A., Tricklebank, M., Steckler, T., 2009. Removing obstacles in neuroscience drug discovery: the future path for animal models. Neuropsychopharmacol 34, 74–89.
Miller, I.W., Norman, W.H., 1979. Learned helplessness in humans: a review and attribution-theory model. Psychol. Bull. 86, 93–118.
Muller, J.M., Morelli, E., Ansorge, M., Gingrich, J.A., 2011. Serotonin transporter deficient mice are vulnerable to escape deficits following inescapable shocks. Genes, Brain Behav. 10, 166–175.
Murphy, D.L., Lesch, K.-P., 2008. Targeting the murine serotonin transporter: insights into human neurobiology. Nat. Rev. Neurosci 9, 85–96.
Palermo-Neto, J., de Oliveira Massoco, C., de Souza, W.R., 2003. Effects of physical and psychological stressors on behavior, macrophage activity, and Ehrlich tumor growth. Brain Behav. Immun. 17, 43–54.
Phelps, E.A., LeDoux, J.E., 2005. Contributions of the amygdala to emotional processing: from animal models to human behavior. Neuron 48, 175–187.
Pryce, C.R., Azzinnari, D., Spinelli, S., Seifritz, E., Tegethoff, M., Meinlschmidt, G., Helplessness: a systematic translational review of theory and evidence for its relevance to understanding and treating depression. Pharm. Ther., in press doi:10.1016/j.pharmthera.2011.06.006.
Randich, A., LoLordo, V.M., 1979. Associative and nonassociative theories of the UCS preexposure phenomenon: implications for Pavlovian conditioning. Psychol. Bull. 86, 523–548.
Robbins, T.W., 2005. Controlling stress: how the brain protects itself from depression. Nat. Neurosci. 8, 261–262.
Seligman, M.E., 1972. Learned helplessness. Annu. Rev. Med. 23, 407–412.
Siegmund, A., Dahlhoff, M., Habersetzer, U., Mederer, A., Wolf, E., Holsboer, F., Wotjak, C.T., 2009. Maternal inexperience as a risk factor of innate fear and PTSD-like symptoms in mice. J. Psychiatr. Res. 43, 1156–1165.
Willner, P., Mitchell, P.J., 2002. The validity of animal models of predisposition to depression. Behav. Pharmacol. 13, 169–188.